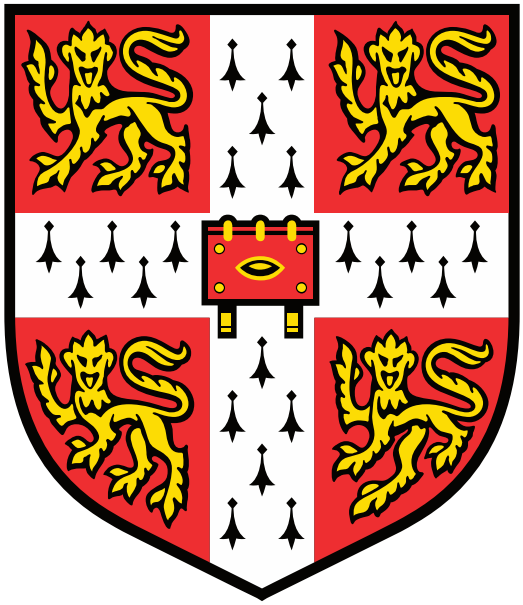
An investigation into whether an exercise intervention during pregnancy can prevent the programming of cardiovascular disease in the offspring of obese mothers

Jessica Holly Beeson



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Summary

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A strong body of evidence suggests that environmental insults from the point of fertilisation to birth and neonatal life can shape the health of the individual for many years to come. Adverse exposures, such as maternal overnutrition, in the early life environment increase the risk of traditionally adult-onset diseases such as cardiovascular disease and type 2 diabetes adding greatly to the next generation's burden of disease. Studies in animal models provide strong evidence that these effects are mediated by non-genetic *programmed* mechanisms. This is of particular concern, as recent studies in the UK suggest that over half of women are now overweight or obese during pregnancy. Current preventative strategies for adult cardiovascular disease have, thus far, focused on reducing an individual's modifiable risk factors. However, given growing evidence that risk of cardiovascular disease is determined *in utero*, there is strong rationale that disease risk from mother to child could be reduced prior to birth, through targeted interventions in the mother before and during pregnancy.

Using an established murine model of maternal diet-induced obesity during pregnancy, the first aim of this thesis was to characterise potential programming factors in the obese mother and identify those that were targeted by a treadmill exercise intervention. Through feeding of an obesogenic diet, dams became heavier, with increased fat mass, and showed insulin resistance at weaning. Previous work has shown the intervention improved maternal insulin sensitivity during pregnancy (E19) and data from this thesis revealed that this was not accompanied by any changes to body composition.

Previous data using this model showed that male offspring born to obese dams have pathological cardiac hypertrophy and *ex vivo* cardiac dysfunction. A second aim of this thesis was to establish if exercise intervention in obese dams was protective to the cardiovascular health of the offspring. These studies revealed that maternal exercise intervention during obese pregnancy had a positive impact by preventing pathological left ventricular cardiac hypertrophy and *in vivo* dysfunction, but did not prevent programmed hypertension in the

male offspring. This demonstrates that offspring cardiac hypertrophy and dysfunction can be programmed independently of hypertension by maternal diet-induced obesity.

The third aim of this thesis was to establish how female offspring were impacted by maternal obesity. The results demonstrated that female offspring born to obese dams were hypertensive and displayed right ventricular cardiac hypertrophy. However, there was no observable effect of maternal obesity on cardiac function in female offspring at this age. This highlights the potential sexually dimorphic effects of developmental programming by maternal obesity.

A final aim of this thesis was to assess the immediate consequences of maternal obesity on the fetal heart and whether maternal exercise had any impact. This showed that in late gestation (E19), cardiac remodelling were already present in the male fetuses of obese dams, and the exercise intervention did not fully prevent this adverse finding.

In conclusion, this thesis highlights that the cardiovascular health legacy of an individual is determined by maternal nutrition before birth and by the intrauterine environment. Just a small improvement in offspring risk could have important implications for the future prevalence of cardiovascular disease worldwide. Importantly this thesis highlights a potential need for combination intervention strategies to tackle the epidemic of obesity in pregnancy, as maternal exercise alone was not sufficient to reduce all aspects of the future burden of cardiovascular disease.

Declaration

The research described in this thesis was conducted in the Department of Clinical Biochemistry at the University of Cambridge. This thesis is the result of my own work and any other work carried out in collaboration has been acknowledged accordingly. This thesis has not been submitted for any other degree, diploma or other qualification and excluding figures and bibliography does not exceed 60,000 words.

Signed: Date:

Jessica Holly Beeson

Publications

Loche E, Blackmore HL, Carpenter, AA, **Beeson JH**, Pinnock P, Ashmore TJ, Aiken CE, De Almeida-Faria J, Schoonejans JM, Giussani DA, Fernandez-Twinn DS, Ozanne, SE (2018) Maternal Diet-Induced Obesity Programmes Cardiac Dysfunction in Male Mice Independently of Post-Weaning Diet. *Cardiovascular Research*, Aug;(114)10:1372-1384

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Abstracts

2015 Cambridge metabolic network- why is exercise good for us? - poster

2016 MRL student symposium, Cambridge – poster

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2017 Cambridge Cardio-Metabolic Event – talk

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Abbreviations

ACOG: American College of Obstetricians and Gynecologists

AFE: Atwater fuel energy

ANOVA: Analysis of variance

ApoE: Apolipoprotein E

APS: Ammonium persulphate

ARC: Arcuate nucleus of the hypothalamus

Asc Ao: Ascending aorta

AWERB: Animal Welfare and Ethical Review Body

BMI: Body mass index

BP: Blood pressure

Bpm: Beats per minute

BSA: Bovine serum albumin

BW: Bodyweight

Ca^{2+}: Calcium

Cat: Catalase

cDNA: Complementary DNA

CO_2: Carbon dioxide

CVD: Cardiovascular disease

dH_20: Distilled water

DOHaD: Developmental Origins of Health and Disease

DTT: Dithiothreitol

E: Embryonic day

ECG: Electrocardiogram

EDV: End diastolic volume

Eef1e1: Eukaryotic translation elongation factor 1 epsilon 1

Ehd2: EH domain containing 2

EKV^{TM}: ECG-gated Kilohertz Visualization

ELISA: Enzyme linked immunosorbent assay

EMPOWaR: Effect of metformin on maternal and fetal outcomes in obese pregnant women

ESV: End systolic volume

ETIP: Exercise Training in Pregnancy

FFA: Free fatty acid

FGR: Fractional growth rate

GDM: Gestational Diabetes

GI: Glycemic index

Gpx3: Glutathione peroxidase

GTT: Glucose tolerance test

GWG: Gestational weight gain

HF: Heart failure

HFD: High fat diet

HFpEF: Heart failure with preserved ejection fraction

HOMA-IR: Homeostatic model assessment for insulin resistance

HR: Heart rate

i.p.: intraperitoneal

IUGR: Intrauterine growth restriction

IVS: Interventricular septum

LGA: Large for gestational age

LL: Left lumen

LV: Left ventricle

LVID: Left ventricle internal diameter

LVPW: Left ventricle posterior wall

MiG: Metformin in gestational Diabetes

MOP: Metformin in Obese non-diabetic Pregnant women

MYC: Myosin heavy chain

Myh6: Myosin heavy chain polypeptide 6, cardiac muscle (alpha)

Myh7: Myosin heavy chain polypeptide 7, cardiac muscle (beta)

NAFLD: Non-alcoholic fatty liver disease

NCD: Non-communicable disease

NHP: Non-human primates

NHS: National Health Service

NICE: National Institute for Health and Care Excellence

NO: Nitric oxide

Nppa or ANP: Atrial natriuretic peptide

Nppb or BNP: Brain natriuretic peptide

NPR-A: Guanylyl cyclase-linked natriuretic peptide receptor-A

Nrf2: Nuclear factor erythroid 2–related factor 2

Ob-Ex: Obese-exercised

PCR: Polymerase chain reaction

Phos-Troponin I: Phosphorylated Troponin I

PND: Postnatal day

RL: Right lumen

RNA: Ribonucleic acid

ROS: Reactive oxygen species

Rpl4: Ribosomal protein L4

RT: Reverse transcriptase

RV: Right ventricle

SBP: Systolic blood pressure

SDS: Sodium dodecyl sulphate

SDS PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SV: Stroke volume

PBS: Phosphate buffered saline

PVDF: Polyvinyl difluoride

SEM: Standard error of the mean

SERCA2: Sarcoplasmic reticulum calcium ATPase

SGA: Small for gestational age

Sod2: Superoxide dismutase 2

SR: Sarcoplasmic reticulum

TEMED: Tetramethylethylenediamine

T2D: Type 2 diabetes

Tbp: TATA binding protein

TBS: Tris-buffered saline

TD-NMR: Time domain nuclear magnetic resonance

TIFF: Tagged image file format

TRE: Thyroid hormone responsive element

UPBEAT: UK Pregnancies Better Eating and Activity Trial

v/v: Volume by volume

w/v: Weight by volume

w/w: Weight by weight

WHO: World Health Organisation

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1. General Introduction

1.1 Cardiovascular Disease (CVD)

The World Health Organisation (WHO) published a global action plan in 2008 to reduce the preventable and avoidable burden of morbidity, mortality and disability associated with non-communicable diseases (NCDs). The target was to prioritise NCDs and their prevention by reducing modifiable risk factors through the creation of a health-promoting environment, while enabling research to focus on the prevention. An estimated 36 million deaths, equating to 63% of the 57 million deaths that occurred globally in 2008, were caused by NCDs, comprising mainly of cardiovascular diseases (CVDs) (48% of NCDs), cancers (21%), chronic respiratory diseases (12%) and diabetes (3.5%) (WHO, 2013).

1.1.1 Incidence of CVD and risk factors

In 2012, CVD was the most common cause of death in the UK for women (28% of all female deaths), but for men cancer is now the most common cause of death (32% of all male deaths) with CVD being the second most common (29% of all male deaths) (Bhatnagar *et al.*, 2015). Furthermore, over one quarter of premature deaths (before the age of 75 years) in men and women were caused by CVD (WHO, 2013). Treatments and advances in surgical techniques mean that the chance of survival after a first CVD event have increased, but the patient is likely to live with the repercussions for the rest of their life (Bhatnagar *et al.*, 2016). Employing prevention strategies before the progression into disease is critically important to help alleviate the disease burden.

The major risk factors for CVD are age, diabetes, hypertension, hypercholesterolaemia, tobacco use, physical inactivity and an unhealthy diet, with the last two often leading to the onset of obesity. These are all modifiable risk factors, meaning that with lifestyle changes they can be eliminated, drastically decreasing the risk of CVD. It has been suggested that at least 80% of premature deaths from heart disease and stroke could be avoided through healthy diet, regular physical activity and avoiding tobacco smoke (WHO, 2017). These risk factors are still globally prevalent; obesity is a worrying problem with its growth now classified as an epidemic (see Section 1.2). Other diseases/disorders also increase the risk of CVD, for example hypertension and hypercholesterolemia, and so by successfully managing these conditions the CVD risk is lowered. Beta-blockers, angiotensin-converting enzyme inhibitors

(blood pressure-lowering drugs), and statins (cholesterol-lowering drugs) independently work to reduce the risk of recurrent vascular events by 75% when combined with cessation of smoking (WHO, 2017).

Age is a non-modifiable risk factor for CVD, and with an ageing population due to increased life expectancy, this will become a challenge. Recently the influence of genetic risk factors in predisposing individuals to CVD has become more understood. Key polymorphisms have been linked with the onset of CVD, such as genetic differences in genes that cause high blood pressure (BP) (Wang and Staessen, 2000), severe hypercholesterolemia (Abifadel *et al.*, 2003), hypertrophic cardiomyopathy (Olson *et al.*, 2000), and atrial or ventricular septal defects (Garg *et al.*, 2003).

1.1.2 Economic burden of CVD

The National Health Service (NHS) in England spent around £6.8 billion on CVD in 2012/2013, the majority of which was spending on secondary care. There were over 1.6 million episodes related to CVD in NHS hospitals, accounting for 10.1% of all inpatient episodes among men and 6.3% among women. It is clear that CVD remains a substantial health and economic burden despite the significant decline in incidence and mortality that has already been achieved (Bhatnagar *et al.*, 2016). The decreased mortality rate of CVD means that more people are surviving and living with the implications of their disease, resulting in an increased demand in secondary care needed to manage their conditions. It has therefore been noted that the cost of inaction is far greater than the cost of taking action to prevent CVD and other NCDs (WHO, 2013).

1.2 Obesity Epidemic: prevalence and impact

Understanding the cause and consequence of obesity is essential for healthcare systems worldwide. Excess bodyweight is the sixth most important risk factor contributing to the overall burden of disease worldwide (Ezzati *et al.*, 2002). This sits behind other risk factors that are often associated with obesity, such as high cholesterol levels (3rd) and low intake of fruit and vegetables (5th). The WHO describes obesity as a global epidemic and a highly prevalent but neglected public-health problem (WHO, 2000), and in fact worldwide obesity has more than doubled since 1980. In 2014, 39% of adults worldwide were overweight and of these, 13% of adults were obese (Figure 1.1). Body size can be assessed using a variety of measures, including weight, height, and waist circumference. A widely utilized tool is body

mass index (BMI) which defines normal weight as a BMI of 18.5–24.9 kg/m^2, overweight as a BMI of 25–29.9 kg/m^2, and obesity as a BMI of 30 kg/m^2 or greater (WHO, 2015).

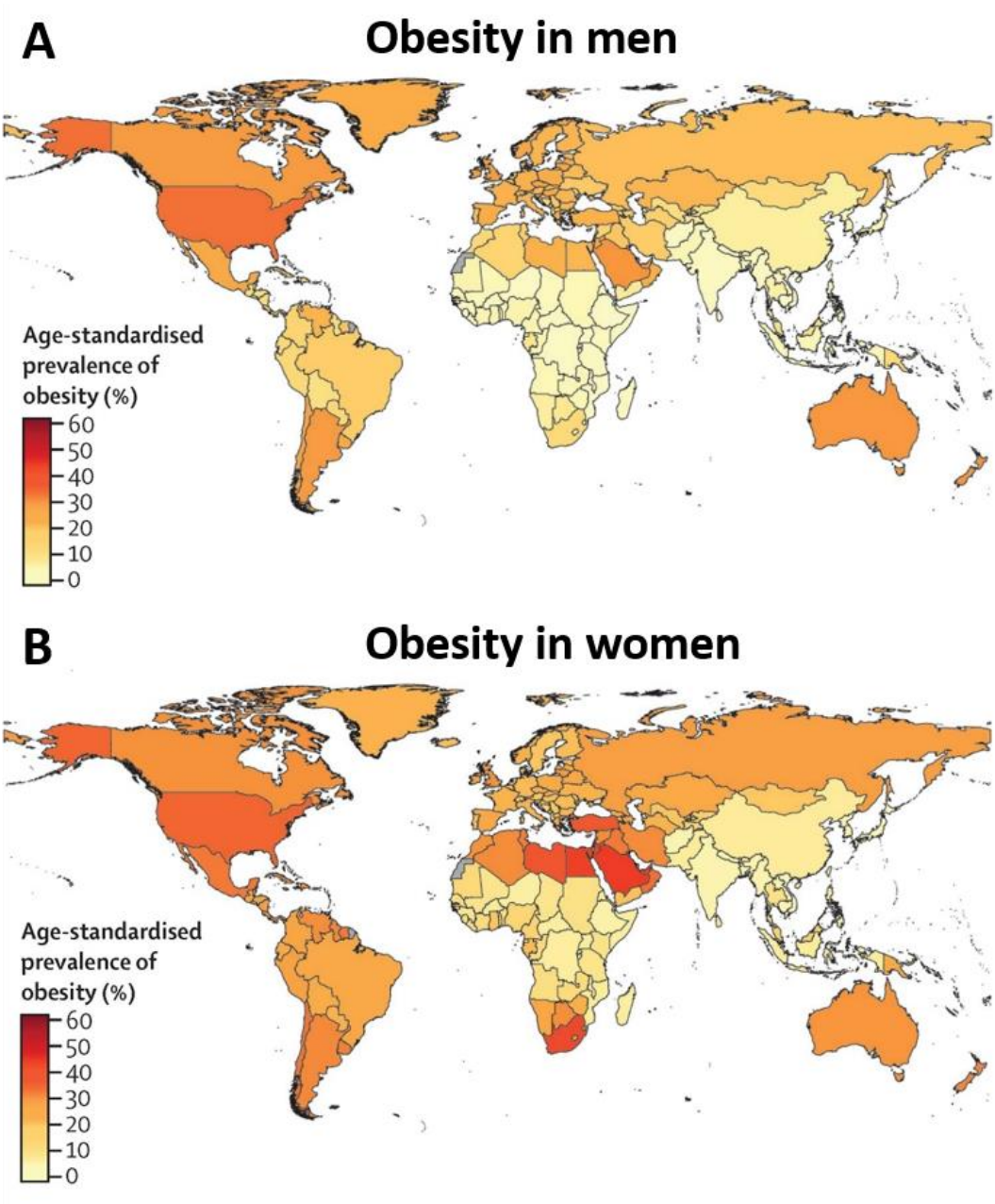


FIGURE 1.1: Prevalence of obesity worldwide. Global heat map in 2014 of obesity prevalence in A) women and B) men. Adapted with permission [CC BY] (NCD Risk Factor Collaboration, 2016).

Obesity is a chronic condition that is often progressive, despite it being preventable, and is multifactorial in its cause. Levels of obesity are thought to be rising, at least in part, due to increased saturated fat and simple carbohydrate intake, combined with physical inactivity. It has been suggested that if the trends in obesity prevalence continue, the global obesity target

(to halt the rise in obesity by 2025 to 2010 levels) will be almost impossible to achieve (NCD Risk Factor Collaboration, 2016).

Studies assessing the genetic contribution to the obesity epidemic suggest that any identified loci have a fairly modest effect size so far. FTO has the largest impact in people from European descent but still only increases BMI by 0.26– 0.66 kg/m^2 (Loos, 2009). This still leaves a large proportion of increases in bodyweight unexplained. Despite the large number of genome-wide association studies, the obesity-associated variants only represent 3% of predicted BMI heritability and cannot explain the rapid increase in obesity prevalence (Albuquerque *et al.*, 2017).

Obesity is a national health burden and in the US it now outranks both smoking and drinking in its deleterious effects on health and healthcare costs (Sturm, 2002). Many of the co-morbidities of obesity relate to metabolic syndrome, defined by the WHO as obesity, hypertension and dyslipidemia with or without hyperglycemia (Alberti and Zimmet, 1998). Current calculations suggest that obesity at the age of 40 will decrease your life expectancy by seven years (Peeters *et al.*, 2003). An analysis has been carried out to estimate the number of years of ill health and number of lives lost between ages 30-75yrs due to excess weight. The results showed that ischaemic heart disease causes the greatest number of years lost to disability, with diabetes a close second (Haslam and James, 2005).

1.3 Developmental Origins of Health and Disease (DOHaD)

The traditional risk factors of CVD, including the modifiable risk factors currently being targeted by the WHO, focus on the current environment or lifestyle that the individual is experiencing. It is now thought that our risk of CVD may actually be determined *in utero* through the maternal environment. This changes the traditionally held view in the clinic and highlights another key period of life where preventative strategies should be applied. Starting in the early 1990s, low birthweight and/or poor fetal growth have been associated with poor health in later life (e.g. abdominal fatness, CVD and increased blood pressure) (Barker, Osmond, *et al.*, 1989; Barker, Winter, *et al.*, 1989; Law *et al.*, 1992; Barker *et al.*, 1997). Alongside genetics and environmental/lifestyle factors, these studies introduced the idea of a third factor in the development of poor cardiometabolic health: the influence of the early life environment and in particular maternal/fetal nutrition. Both maternal malnutrition and maternal obesity are considered to be suboptimal intrauterine environments and act as large

influencers on the health and disease of their offspring. Their impact will be explored in the following sections of this introduction.

The study of DOHaD aims to find the mechanisms by which the environment experienced during fetal development contributes to the risk of disease in postnatal life. Developmental periods are sensitive to environmental stimuli as the system has an element of plasticity; this mainly occurs *in utero* but can stretch into the postnatal period with the exact window and duration dependent on the species and tissue type. It is proposed that changes in response to the maternal environment during the critical period of fetal development will have long-lasting effects on the future health of that individual, or in other words will 'programme' the individual to be predisposed to disease (Figure 1.2).

1.3.1 Thrifty Phenotype hypothesis

The original hypothesis, based on associations of low birthweight and type 2 diabetes (T2D)/CVD that started this field was the Thrifty Phenotype hypothesis, proposed in the 1990s (Hales and Barker, 1992). The hypothesis describes the idea that maternal malnutrition causes changes that preferentially divert nutrients and/or oxygen supply to essential organs (e.g. brain) of the fetus at the expense of the non-key organs (e.g. adipose tissue and pancreas). These changes are likely beneficial in the short-term in continued conditions of poor postnatal nutrition, but the resulting altered birth phenotype may be maladaptive in later life, especially in conditions of postnatal overnutrition thus contributing to the adverse health outcomes. The fetus was proposed to adapt its metabolism to expect a similarly depleted environment postnatally through efficient storage of nutrients. Inappropriate control points are then established, such as decreased physiological tolerance to increased adiposity and a rich diet, thus disturbing the metabolic balance throughout the life of the individual in conditions of overnutrition (Rees, McNeil and Maloney, 2008) (Figure 1.2).

The Thrifty Phenotype hypothesis was based on observations from a study of Hertfordshire males aged 64 whose birth records were available. There was an association between low birthweight and increased systolic blood pressure (SBP), increased prevalence of atherosclerosis (Barker, Osmond, *et al.*, 1989; Barker, Winter, *et al.*, 1989) and increased prevalence of T2D (Hales et al., 1991). This cohort was also used to draw associations of continued low bodyweight at one year with metabolic disease (including impaired glucose tolerance and increased incidence of T2D (Hales *et al.*, 1991)). The strong association of low birthweight and poor health has been replicated in different populations worldwide.

1.3.2 Predictive Adaptive Response hypothesis

The Predictive Adaptive Response hypothesis, which followed the Thrifty Phenotype hypothesis, proposed that changes will occur to promote fetal survival. These changes may not be immediately beneficial to the developing fetus, but if there is a correct prediction of postnatal environment it may become advantageous in later life. If the postnatal conditions do not match the prediction made *in utero* then this will result in a detrimental 'mismatch' and ill health will likely develop (Godfrey *et al.*, 2007). This model goes beyond maternal undernutrition and extends to include maternal overnutrition. Rapid 'catch up growth' can occur in offspring who are born small for gestational age (SGA); this causes an increased risk of T2D and CVD morbidity and mortality, likely driven by the subsequent environmental mismatch (Barker, 2002; Barker *et al.*, 2005). Since the rate of postnatal growth determines the risk of disease in the offspring, the impact of the programming time window could extend beyond birth.

Early life programming is thought to play an important role in the aetiology of obesity, T2D, and CVD. The importance of tackling such NCDs has been recognised by the recent WHO global action plan (WHO, 2013), however the importance of a poor intrauterine environment and its contribution to the current NCD epidemic requires further study.

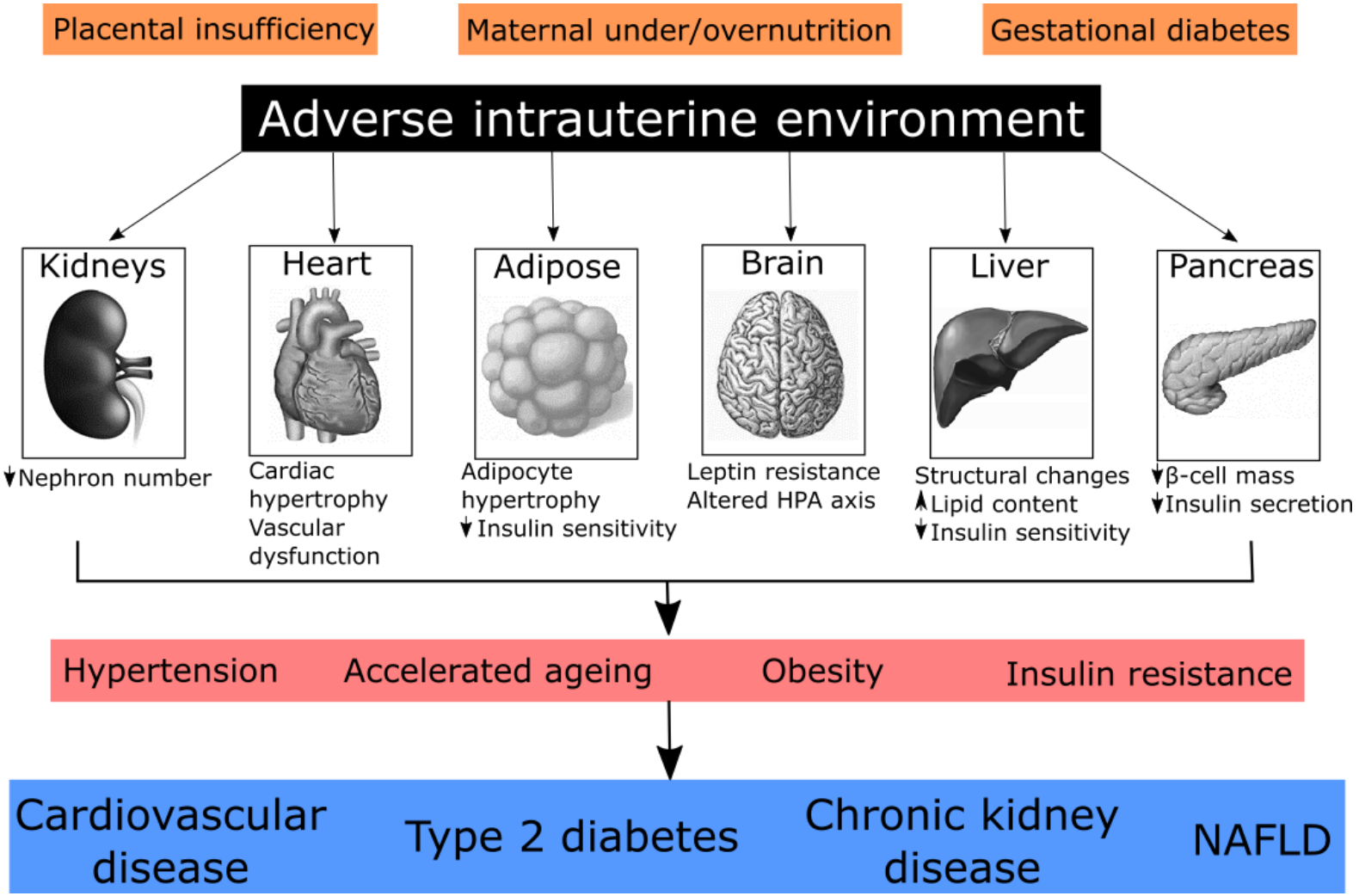


FIGURE 1.2: Developmental Origins of Health and Disease. The multi-organ impact of an adverse intrauterine environment and the subsequent health burden on the offspring. NAFLD- Non-alcoholic fatty liver disease.

1.4 Maternal undernutrition: evidence from human cohorts

1.4.1 Dutch Hunger Winter famine

Famine is a problem that is confined more to the developing world, with less than 2% of the total number of people suffering from undernourishment coming from a developed country (FAO, 2015). Valuable data from a human cohort came from analysing data from births during the Dutch Hunger Winter famine, a period of five months of malnutrition in the western part of the Netherlands in the winter of 1944-45. During adulthood, children born to mothers who experienced the famine during early pregnancy had increased levels of CVD, increased BMI and were glucose intolerant (Ravelli *et al.*, 1999; De Rooij *et al.*, 2006). Children born to mothers who were pregnant before the famine and experienced famine in the later stages of pregnancy were also born with low birthweight and with an increased risk of obesity and T2D, but these individuals had a milder cardiovascular phenotype (Ravelli *et al.*, 1999). This important human cohort provided some of the first evidence that a suboptimal *in utero* environment for development has an impact on the health burden of the now adult offspring. It also highlights the importance of the timing of an insult during development, with malnutrition during early pregnancy causing the most harm.

Similar outcomes has been shown in those exposed to other famines *in utero*, including famines in Austria following the two world wars (1918 and 1946-47) (Thurner *et al.*, 2013). The Chinese famine (1959-1961) has shown that exposure to severe famine conditions in fetal life caused an increased risk of hyperglycemia and dyslipidemia in adulthood (Li *et al.*, 2011; Z. Wang *et al.*, 2017). Exposure to the famine specifically in the first trimester increased the risk of hypertension in adulthood (Wang *et al.*, 2016).

1.4.2 Twin Studies

A critical tool in the field of developmental programming is using discordant siblings in studies to address and limit contributions by genetic and current environmental factors. The validity of using twin studies to make conclusions about singleton pregnancies has been questioned, due to the differences in the normal intrauterine growth profiles. However, studies between twins discordant for T2D showed that the twin with the lower birthweight is more glucose intolerant and has an increased risk of T2D. Low birthweight, due to inadequate energy and/or nutrient intake, prevents the developing fetus from reaching a healthy size and is associated with increased risk of disease (Ijzerman, Boomsma and Stehouwer, 2005).

1.5 Programming by maternal undernutrition in animal models

Animal studies permit better control of variables while generating data in a shorter time frame. They also allow a greater investigative burden on mothers and offspring than tolerated by humans, and this allows causative associations to be made (Rabadan-Diehl and Nathanielsz, 2013). Human epidemiology alone cannot easily dissect the cause-effect relationships, while animal studies can identify the specific mechanisms involved. Laboratory animals are kept in tightly controlled conditions, allowing any confounding factors identified by epidemiological studies to be removed, to adequately assess the alterations at the molecular and whole-body level.

1.5.1 Maternal nutrient restriction

Models of maternal undernutrition have been achieved by caloric restriction in a range of animal species. One study showed that rats calorically restricted by 50% during pregnancy had smaller pups that showed marked postnatal 'catch up growth', and the offspring had increased intra-abdominal fat at four months of age (Suzuki, Shibanuma and Kimura, 2010). This obesity-prone phenotype has been attributed to a premature leptin surge in neonatal life. The leptin surge is critically important for the formation of energy regulation circuits in the hypothalamus (Yura *et al.*, 2005). In another study, rats received 30% of *ad libitum* intake throughout gestation with offspring displaying hyperphagia, hypertension, and markedly increased plasma insulin and leptin levels. These metabolic and cardiovascular abnormalities were amplified by postnatal hypercaloric nutrition, suggesting this is important in the aetiology of adult-onset disease (Vickers *et al.*, 2000).

Maternal undernutrition will typically result in fetal intrauterine growth restriction (IUGR), and this represents another model used to study maternal undernutrition. In a baboon model of IUGR, juvenile offspring displayed signs of hypertension, insulin resistance and metabolic syndrome (Choi *et al.*, 2011). Cardiac dysfunction has also been detected in IUGR fetuses and neonates (Fouzas *et al.*, 2014). Using cardiac magnetic resonance imaging, it was shown that IUGR caused young adult baboons to have systolic dysfunction and left ventricle remodelling that mimics accelerated ageing (Kuo *et al.*, 2017).

1.5.2 Low protein diet

To determine the impact of maternal macronutrient deficiency, a maternal low protein model was established by Snoeck and colleagues (Snoeck *et al.*, 1990). The model uses a control

20% protein diet compared to an isocaloric 8% protein diet in pregnancy that causes a significant reduction in birthweight (Chen *et al.*, 2009). Endothelial dysfunction, hypertension, and increased cardiac sympathetic tone were seen in the offspring exposed to protein restriction *in utero* (Brawley *et al.*, 2003; Torrens *et al.*, 2009; Barros *et al.*, 2015). ApoE mice fed an isocaloric 8% low protein diet during pregnancy and lactation demonstrated young adult offspring with accelerated development and increased progression of atherosclerotic lesions (Blackmore *et al.*, 2012).

Studying the effects of undernutrition in pregnancy is still relevant in teenage pregnancies and pregnancies in older mothers, where SGA still occurs, as well as in under-developed parts of the globe. Four decades ago the world's underweight population was more than double that of the obese equivalent, however this is no longer true with global statistics showing that more people are obese than underweight (NCD Risk Factor Collaboration, 2016). With this in mind, the importance of studying maternal obesity has never been so important with an estimated 50% of childbearing aged women (25-34 years old) being overweight or obese in the UK (Davies *et al.*, 2014). As well as immediate detrimental consequences to mother and child, there is also now growing evidence to suggest that exposure to an obesogenic environment *in utero* programmes poor cardiometabolic health.

1.6 Maternal obesity

The global obesity epidemic is affecting all groups within the population, including women of childbearing age. A large retrospective study among maternity services in England showed the incidence of first trimester obesity (30 kg/m^2) has increased from 7.5% to nearly 15.6% of the pregnant population over an 18 year period (Heslehurst *et al.*, 2010).

1.6.1 Risks to the mother

Obesity during pregnancy increases immediate adverse pregnancy outcomes such as stillbirth, pre-eclampsia and caesarean delivery (Cedergren, 2004; Athukorala *et al.*, 2010; Crane *et al.*, 2013). These increased risks are independent of partner status, smoking or maternal age. Incidence of fetal distress and a low APGAR score (score for physical condition of a newborn) increases with maternal obesity (Cedergren, 2004).

Pre-eclampsia is characterised clinically by high maternal BP, proteinuria, and oedema and makes up 3-7% of all pregnancy hypertensive disorders (Pivarnik *et al.*, 2006). Women with a history of pre-eclampsia are more likely to go on to develop essential hypertension in the

years that follow, creating a further health burden (Nisell *et al.*, 1995). In fact, pre-eclamptic women often experience metabolic disturbances that mirror those of a non-pregnant CVD patient, such as hyperlipidemia, oxidative stress, and sympathetic over-activation, despite having no CVD diagnosis for themselves (Pivarnik *et al.*, 2006).

Caesareans due to ineffective intrauterine contractility are more common with increasing maternal BMI (Cedergren, 2009). Leptin, a hormone that increases with percentage body fat, has an inhibitory effect on myometrial contractions (Moynihan *et al.*, 2006). Macrosomia (a newborn significantly larger than average) and the narrowing of the maternal pelvis due to increased soft tissue mass can cause cephalopelvic disproportion (failure of the fetus to travel through the birth canal). This is thought to be one explanation for the increased need for caesarean delivery in obese pregnancies (Ehrenberg, Huston-Presley and Catalano, 2003; Sherrard *et al.*, 2007). The risks of maternal obesity to the mother are summarised in Figure 1.3.

Insulin resistance is a normal part of pregnancy and is very important in maintaining fuel supply to the growing fetus, however many obese women enter pregnancy with pre-existing insulin resistance which worsens throughout gestation (Catalano *et al.*, 1999). The risk of developing gestational diabetes mellitus (GDM) is two times higher in women who are obese during their pregnancy compared to normal weight women (Athukorala *et al.*, 2010). In addition, obese women diagnosed with GDM more often require insulin to control blood glucose level than normal weight women who can often successfully use diet to control the condition (Langer *et al.*, 2005). Differences in metabolic profile, including dyslipidemia, are present in obese pregnant women at least ten weeks prior to diagnosis of GDM (White *et al.*, 2017). Both maternal obesity and GDM have adverse pregnancy outcomes, for example both independently increase the risk of the need for caesarean section (Ehrenberg *et al.*, 2004). Studies have shown that pre-pregnancy BMI may have stronger influence on caesarean section, pregnancy-induced hypertension and large for gestational age (LGA) baby than GDM (Ricart *et al.*, 2005), however the combination has a more severe outcome on maternal and neonatal outcomes then either alone (Catalano *et al.*, 2012; Wahabi *et al.*, 2014).

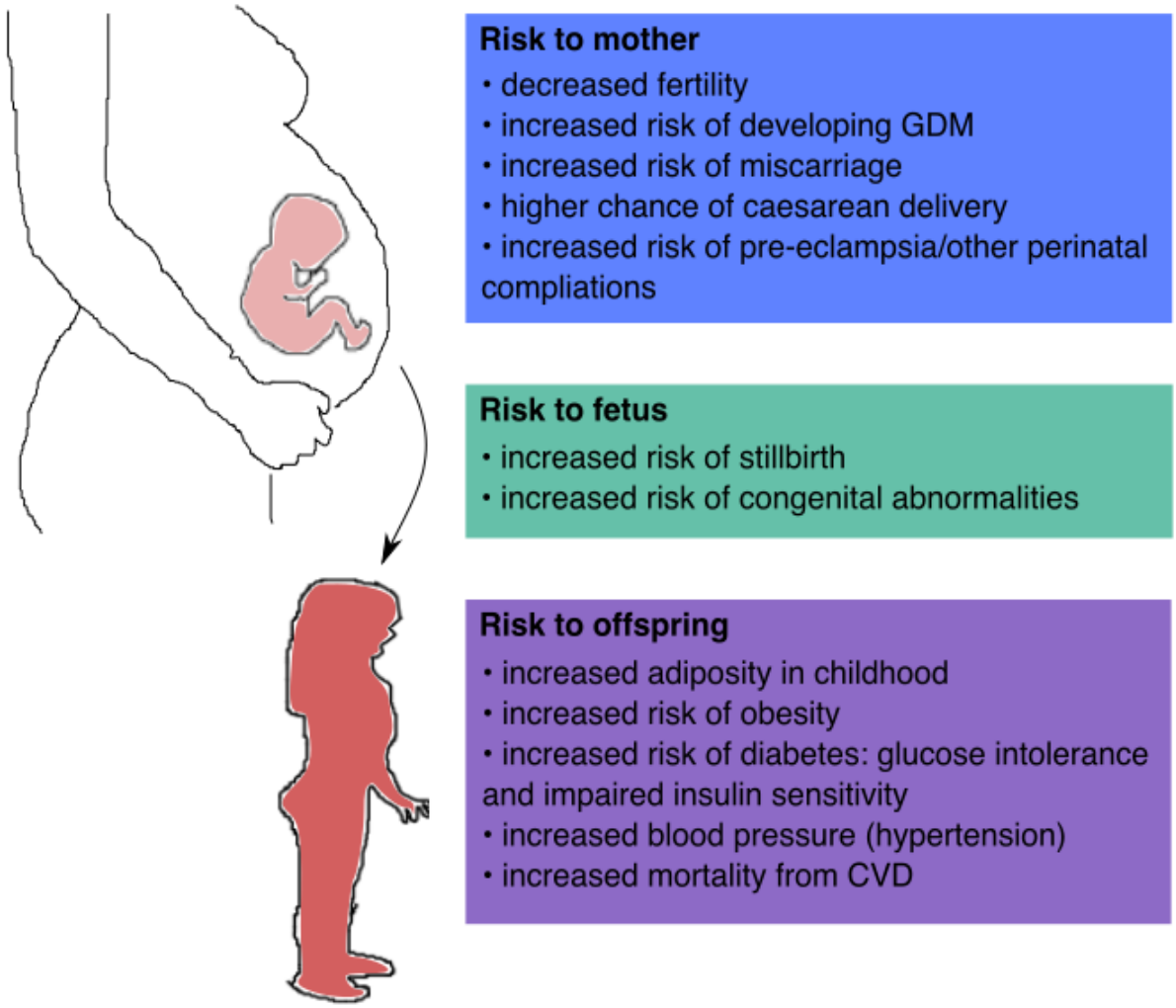


FIGURE 1.3: Summary of the wide-ranging consequences of maternal obesity. The consequences not only have an immediate impact on the mother and her pregnancy but also have programmed effects on the offspring's health.

1.7 Evidence from human cohorts for programming by maternal obesity

1.7.1 Association studies

The weight of a fetus is influenced by the parents' weights and heights through a genetic link, but maternal weight has an additional programming influence through the intrauterine environment. Obesity during pregnancy has generally been assumed to be associated with offspring born LGA, however research now suggests obese mothers are just as likely to have offspring born SGA (Rajasingam *et al.*, 2009), presumably in part related to effects of maternal obesity on placental function. In contemporary populations, where obesity prevalence is high, the relationship between risk of developing adulthood disease and birthweight is a U-shaped curve, with both low and high birthweight offspring at equal risk of cardiometabolic dysfunction (Pettitt and Jovanovic, 2001). These results show that exposure

to obesity during development can produce offspring in both birthweight groups with increased risk of disease, suggesting a growing threat to future generations. Maternal obesity is a heterogeneous condition and can be accompanied by metabolic syndrome alongside varying degrees of hypertension, insulin resistance, and dyslipidemia. It is, therefore, likely to present challenges in the study of its impact on fetal programming as the vulnerable fetus will be exposed to a myriad of different maternal "programming factors" that also may vary between individuals.

1.7.2 Helsinki Birth Cohort

The Helsinki Birth Cohort study was a unique undertaking, collecting extensive epidemiological data on 13,345 individuals throughout their lifespan, including prenatal life, childhood and later life. Maternal BMI correlated with many different offspring health outcomes including premature death, with the strongest effect on CVD and T2D (Eriksson *et al.*, 2014). An association between maternal obesity and atrial fibrillation, a significant risk factor for future mortality from CVD, has also been demonstrated in this cohort (Odutayo *et al.*, 2016; Johnson *et al.*, 2017).

1.7.3 Adoption studies

Often it is difficult to show to what extent associations between parental and offspring adiposity is explained by shared postnatal environmental and lifestyle factors, but this can be studied in adoption studies. Despite adopted offspring being exposed to the shared environmental and lifestyle factors of their adoptive family, their BMI is still more strongly associated with that of their biological parents (Sorensen *et al.*, 1992). This suggests that the mediator could be genetic and/or programmed.

1.7.4 Sibling pair studies in Pima Indians

Sibling studies are useful as you are able to control for genetics and lifestyle, as much as possible, by comparing siblings born to the same parents and raised in the same household. A population in central America known as the Pima Indians has one of the largest incidences of T2D and obesity; 50% of adults aged over 35 years have T2D (Knowler *et al.*, 1981). Studies using siblings born before and after the mother was diagnosed with diabetes enabled researchers to separate the effects of programming by a diabetic pregnancy and the genetic influence. Siblings born from a diabetic pregnancy had increased prevalence of T2D and an increased BMI when compared to siblings born before the onset of maternal diabetes. Exposure to diabetes during pregnancy therefore delivers a high risk for the development of

diabetes and obesity in offspring which is in excess of risk attributed to transmission of genetic variants alone (Dabelea *et al.*, 1998, 2000).

1.7.5 Siblings studied before and after bariatric surgery

One very powerful approach compares siblings born before and after the mother has undergone biliopancreatic bypass surgery, which causes weight loss and an improvement in maternal metabolic health (Umeda *et al.*, 2011; Maggard-Gibbons *et al.*, 2013). Offspring born after mothers underwent surgery are 52% less likely to become obese when compared to siblings born before surgery (Kral *et al.*, 2006). Offspring born after weight-loss surgery also had improved fasting insulin and homeostatic model of insulin resistance (HOMA-IR) as well as lower BP (Guénard *et al.*, 2013). In general, the sibling born before the weight loss surgery exhibited more indicators of cardiometabolic dysfunction than their sibling (Smith *et al.*, 2009). This study presents critical evidence that the effects are 'programmed' and are not simply genetic since environmental insults from the point of fertilisation to birth have impacted the future health of that individual.

1.8 Risks to the offspring: Human studies

1.8.1 Metabolic dysfunction

There is growing evidence to suggest that pre-pregnancy BMI is a strong predictor of future offspring disease (Parsons, Power and Manor, 2001; Boney *et al.*, 2005). Pre-pregnancy obesity causes an early tendency for offspring to be overweight which is perpetuated as the child grows (Salsberry and Reagan, 2005). Maternal obesity before pregnancy is associated with increased newborn fat mass, as calculated by skinfold thickness before one day of age (Pacce *et al.*, 2016). Furthermore, maternal obesity is associated with an increased risk of obesity and metabolic dysfunction in the offspring and this effect is seen from childhood to adulthood (Boney *et al.*, 2005; Cooper *et al.*, 2010).

A population-based prospective cohort revealed that pre-pregnancy BMI was positively associated with higher adolescent BMI, waist-to-hip ratios, and insulin, glucose and HOMA-IR levels (Gaillard *et al.*, 2016). Insulin resistance programmed at an early age will become more pronounced with age which could ultimately lead to metabolic disease (Rees, McNeil and Maloney, 2008). Maternal obesity is associated with increased BMI in children, and a retrospective study has shown that an increase of each BMI unit (z-score) at seven years old corresponded with 10% and 7% higher risk of CVD in males and females respectively. This

relative risk increased with age, in particular the risk doubled in both sexes by the age of 13 (Baker, Olsen and Sorensen, 2007).

Studies have shown that offspring from a GDM pregnancy have higher mean BMI, and this is independent of birthweight (Avgil Tsadok *et al.*, 2011). When maternal pre-pregnancy BMI is included in the model this effect is markedly reduced, suggesting that the effect of maternal GDM is not independent of pre-pregnancy BMI. Some data suggests that maternal insulin resistance may be a more important predictor of infant adiposity then maternal BMI (Shapiro *et al.*, 2015) as insulin resistance can divert excess fuel to fat accretion and fetal growth (Barbour *et al.*, 2011).

1.8.2 Cardiovascular risk factors

Obese women are more likely to have children with congenital heart defects and the risk increases with advancing BMI (Mills *et al.*, 2010). One study detected that fetuses (at 14 weeks gestation) from obese women have reduced left and right ventricle global strain rate, indicating fetal myocardial dysfunction (Ingul *et al.*, 2016).

Some of the first evidence for an early life overnutrition environment and its influence on cardiovascular consequences comes from a Finnish study that showed a positive association between mother's weight and CVD (Forsén *et al.*, 1997). The Aberdeen cohort study also reported that maternal BMI positively correlates with increased risk of hospital admission and premature death from CVD (Reynolds *et al.*, 2013). This cohort used a large database of pregnant women dating from the 1950s. Offspring were born between 1950 and 1976 and their birth records were linked to the general register of deaths and the Scottish morbidity record systems in order to assess all-cause mortality from CVD. The authors found an association between maternal overweight/obesity and increased cardiovascular events in offspring (maintained after adjustment for current socioeconomic status) (Reynolds *et al.*, 2013). This is concerning, given the increasing prevalence of obesity in child bearing women. Another study demonstrated a positive association between maternal BMI and early indicators of CVD risk factors in young children (Labayen *et al.*, 2010). Higher maternal pre-pregnancy BMI was associated with an adverse cardiometabolic profile in adolescence (Gaillard *et al.*, 2016). These offspring also had increased adiposity and their BMI was closely associated with both maternal pre-pregnancy BMI and an adverse cardiometabolic profile. A prospective cohort of children called Project Viva assessed the relationship between pre-pregnancy BMI and cardiometabolic risk in school age offspring (age range six-ten years old). Children of

mothers with greater pre-pregnancy BMI had more overall as well as central fat, greater insulin resistance, dysregulated adipocytokines, elevated inflammatory markers and higher SBP (Perng *et al.*, 2014).

Offspring blood pressure

The effect of maternal programming on offspring BP has been assessed as another cardiovascular risk factor. Higher maternal pre-pregnancy BMI was associated with elevated SBP in adolescents with a mean age of 17 (Gaillard *et al.*, 2016). The authors also assessed how weight gain in early and mid (but not late) pregnancy had a positive association with a range of poor cardiometabolic parameters in offspring, including SBP (Gaillard, 2015)

In 2009, the Institute of Medicine set guidelines for the appropriate weight gain during pregnancy, broken down by pre-pregnancy BMI (Rasmussen *et al.*, 2009). Gestational weight gain (GWG) has been positively correlated with BP and BMI in exposed children at three years of age. SBP was an estimated to be 0.60 mmHg higher per 5 kg of GWG. This increase was reduced to 0.34 mm Hg per 5 kg weight gain after adjusting for child BMI, as the child's size is a major determinant of BP (Oken *et al.*, 2007). In a supporting study of adults at 21 years, there was a positive association between GWG and offspring SBP that trended towards significant (Mamun *et al.*, 2009). The relevance of higher average SBP in early adulthood is positively associated with future CVD (McCarron *et al.*, 2000). The plethora of effects of maternal obesity on offspring health is summarised in Figure 1.3.

1.9 Risks to the offspring: Animal studies

Animal models from a wide range of species aid the study of maternal obesity and enable further understanding of maternal programming processes. Fetal programming research has commonly used rodents due to their placental similarities to humans, short gestation lengths, and their ability to generate large sample sizes. Most differences in using rodents to study fetal programming come from the fact that rodents are born to large litters with further development required postnatally, whereas humans, sheep, and non-human primates (NHP) are delivered in fewer numbers and born with a greater degree of intrauterine development. The critical windows of vulnerability during development therefore vary among species (Rabadan-Diehl and Nathanielsz, 2013). Due to the long-term nature of CVD it is often true that the data on such outcomes in humans will not be known for many years. Animal models are therefore critical to longitudinally determine the cardiometabolic effects on the offspring.

1.9.1 Large animal models

Although less commonly studied, ovine models are used due to the similarities of placental structure and function in humans, and the ability to have repeated sampling of blood and tissue. Ewes are overfed (150% National research council recommendations) to induce obesity at conception, causing macrosomia, hyperglycemia, and hyperinsulinemia in fetuses at mid-gestation (Zhang *et al.*, 2011). Fetuses from a maternal overnutrition model had altered heart stress signalling, including the activation of TGF-β, p38 and Smad pathways, leading to collagen accumulation and fibrosis (Huang *et al.*, 2010). In the same model, fetal hearts from overfed ewes had impaired cardiac function in response to high workloads in an *ex vivo* perfusion set up (Wang *et al.*, 2010). Adult offspring displayed altered growth, adiposity and glucose tolerance (Long, Ford and Nathanielsz, 2011).

NHP models have supported the work in small animal and ovine models. Offspring of obese baboons had placental and fetal profiles that more closely resembled that of a LGA human fetus despite them being of normal weight (Farley *et al.*, 2010). High fat diet feeding in the preconceptual period and throughout pregnancy resulted in smaller fetuses in the third trimester, but they underwent 'catch up growth' postnatally and showed increased adiposity. Irrespective of postnatal diet these offspring gained early-onset excess weight (Grayson *et al.*, 2010).

1.9.2 Rodent models

Rodent models are the most commonly used experimental animal model. High fat diets (HFD) vary in composition and can include diets that have 40% fat (% of kcal) and others that have 60% fat (% kcal).

<u>High fat diet feeding</u>

Offspring from HFD fed rats (45% kcal from fat) during pregnancy and lactation were significantly more obese than the control equivalents, and this was accompanied by hyperinsulinemia and hyperleptinemia in the offspring (Howie *et al.*, 2009). The authors also had a group of females who were fed the HFD from weaning before continuing in pregnancy and lactation. It is of interest to note that a lifetime of high fat nutrition produced a similar offspring phenotype to simply high fat nutrition in pregnancy and lactation alone. This indicates that the preconception diet is independent of these offspring effects.

HFD maternal feeding (40% energy from fat) during pregnancy and lactation in a mouse model reduced offspring responsiveness to the beneficial effects of exercise (i.e. reduction of fat mass and improvement of insulin resistance) (Kasch *et al.*, 2017). This suggests the offspring are not only more prone to becoming obese, but they also seem less able to combat the negative effects and get themselves out of this unhealthy state.

HFD feeding (60% kcal from fat) from weaning in female mice programmes hypertension and adiposity in male offspring at six months of age (Guberman *et al.*, 2013). The authors showed an associated activation of the adipose renin-angiotensin system (an endocrine system involved in BP homeostasis). Male offspring of mothers fed a HFD during pregnancy and lactation showed impaired nitric oxide (NO)-dependent endothelial and vascular function, and this likely mediated the offspring's elevated BP (Gray *et al.*, 2015). In a rat model of HFD feeding (45% kcal from fat) during pregnancy and lactation, offspring displayed endothelial dysfunction in response to acetylcholine (vasodilator) in both sexes, but an increased BP was only shown in the females at six months of age. HFD feeding (60% kcal) in mice for four weeks prior to breeding and during pregnancy also demonstrated increased BP (both systolic and diastolic) in the female offspring (Liang, Oest and Prater, 2009).

Obesogenic diet feeding

HFDs in rodents have been critical in understanding the effects of a HFD alone, however rodents do not generally find HFDs very palatable so it can often be difficult to cause significant bodyweight gain and such diets are not typical of a human western diet consumed in today's society. In order to fully understand the current diet of the 'western' population, it is important to assess the long-term impact of exposure to a diet both high in fat and in simple sugars, which are very palatable, and this led to the development of obesogenic diets.

Another rat model of overnutrition had cafeteria-style high fat feeding for five weeks before mating to induce obesity. The offspring had increased adiposity, insulin resistance, and hyperphagia (Morris and Chen, 2009). Mouse dams fed a high fat and sugar diet for six weeks before mating and continued on the diet for pregnancy and lactation had offspring that displayed hyperphagia, increased adiposity, and endothelial dysfunction at three months of age. A hypertensive phenotype was observed at six months of age (Samuelsson *et al.*, 2008). Offspring of a rat model of maternal obesity had night-time elevations in mean arterial BP compared with control offspring, and this preceded the development of obesity and hyperleptinemia. The study suggested a sympathetic origin of the observed hypertension, and

this was supported by elevated noradrenaline and renin, and the inhibition of hypertension following β-adrenergic blockade (Samuelsson *et al.*, 2010). This suggests the presence of sympathetic dominance and early cardiac dysfunction. Critically, this occurred before the onset of increased offspring adiposity or hyperleptinemia, indicating it arose as a direct consequence of *in utero* and postnatal maternal environments (Samuelsson *et al.*, 2010). It has been suggested that neonatal hyperleptinemia as a result of maternal obesity causes permanent changes in the central melanocortin system which will contribute to the sympathetic mediated hypertension (Samuelsson *et al.*, 2016). Consistent with this idea, experimental hyperleptinemia in neonatal rat pups from postnatal day 9 to 15 resulted in heightened BP in adulthood (Samuelsson *et al.*, 2016).

Our research group uses a model of maternal diet-induced obesity that attempts to mimic the western diet by feeding mice a diet high in fat and sugar (high fat pellet 20% w/w supplemented with sweetened condensed milk 55% sugar w/w). The female mice are fed this obesogenic diet from weaning and for at least six weeks before mating, so that by mating bodyweight was increased; using body composition analysis this increase can be explained by an increase in fat mass and not lean mass. Obesogenic diet-fed mice had an almost three-fold increase in relative fat mass compared to control lean females. This model enables assessment of the effect of obesity in the mother and its programmed effects, and not just the effect of feeding an obesogenic diet. Previous findings in our lab (described in the following section) provide the basis for all work undertaken in this PhD.

Offspring from these obese mothers show pathological cardiac hypertrophy at an early age, as indicated histologically, and molecularly through the re-expression of fetal genes (Fernandez-Twinn *et al.*, 2012). This is followed by *ex vivo* cardiac dysfunction with both systolic and diastolic dysfunction at 12 weeks of age (reduced left ventricular developed pressure and increased left ventricular end diastolic pressure, respectively) as measured by isolated Langendorff heart perfusion. The advantage of the isolated heart set up is the ability to introduce drugs and therefore test the heart's response to parasympathetic and sympathetic agents (e.g. carbachol and isoprenaline respectively). The ratios of sympathetic to parasympathetic chronotropic and inotropic responses were elevated, suggesting sympathetic dominance, which is often seen as an early hallmark for heart disease and failure (Blackmore *et al.*, 2014). Importantly, this cardiac phenotype occurred in the absence of a change in bodyweight and despite the offspring eating a healthy diet from weaning.

The effect of a maternal and post-weaning obesogenic diet on cardiovascular function was assessed. The same model of maternal obesity was used as described above and then offspring from control and obese dams were weaned onto either a control diet or the same obesogenic diet. This study concluded that maternal obesity-exposure caused poor cardiac health and this was of comparable magnitude to current obesity. There was no additive effect of current obesogenic diet, suggesting that the cardiac dysfunction was programmed independently of offspring diet (Loche *et al.*, 2018). This highlights the worrying finding that the impact of obesity during pregnancy on offspring cardiac dysfunction is of a similar magnitude to that due to current obesity. The opposite was true for offspring hyperinsulinemia, hyperleptinemia, increased fat mass, and hypertension; *in utero* and neonatal exposure to maternal obesity and offspring obesity acted synergistically (Loche *et al.*, 2018). These additive effects could continue to develop over time and possible contribute to the worsening cardiac phenotype that would be expected to occur with age. This indicates the importance of an intervention during pregnancy to prevent the transmission of CVD to the offspring.

1.10 Interventions to prevent adverse programming outcomes

It is clear that interventions to tackle CVD and the associated health burden should start *in utero* and not in adulthood when disease pathology is already emerging. Targeting maternal wellbeing during pregnancy could ensure good cardiovascular health for future generations. An intervention to tackle maternal obesity is important since it not only predisposes offspring to developing CVD, but also offspring are more prone to becoming obese, which is an independent risk factor of developing CVD.

A report from the Institute of Medicine (2010) indicates a need for evidence-based interventions that inform and motivate pregnant women to adopt a healthy lifestyle before and during pregnancy. Interventions to combat the unwanted impact of maternal obesity can be behavioural (dietary or lifestyle changes) or pharmacological. Pregnancy is a life event in which women may be more inclined to make behavioural changes (Oken *et al.*, 2003). If appropriate management was provided before and during pregnancy, a behavioural intervention to promote a healthy lifestyle could be effective, and may even result in persistent positive behavioural changes concerning nutrition and physical exercise postpartum.

1.10.1 Maternal exercise interventions

Exercise during pregnancy

Physical activity makes up the cornerstone in the prevention and management of T2D as it is known to adjust the metabolic health of the patient by improving insulin action (Colberg *et al.*, 2010). This makes it an attractive method to improve the metabolic health of an expectant mother and therefore alleviate the adverse intrauterine environment the developing fetus is experiencing. During pregnancy, there are major physiological adaptations to sustain the continuous requirements of the fetus for nutrients and oxygen; this creates a pseudo-diabetogenic state of progressive insulin resistance (Mottola and Artal, 2016; Musial *et al.*, 2016). Maternal glucose utilisation is spared in the peripheral tissues, with insulin action being altered in the liver, skeletal muscle and adipose tissue. This increases blood glucose and amino acid concentrations, allowing more to be available for placental transport. Exercise promotes insulin sensitivity, particularly in the skeletal muscle (Ren *et al.*, 1994; Holloszy, 2005) and evidence suggests vigorous physical activity in mid-pregnancy predicts improved glucose tolerance in normal, overweight and obese women (Medek *et al.*, 2016).

Some women are apprehensive about exercising during pregnancy due to the perceived ill effects that this will bring, however moderate aerobic exercise during a healthy pregnancy has been shown to have no detrimental effects on the mother's health (Clapp, 1991; Platt *et al.*, 2013). It is well known that exercise improves cardiovascular function (Gielen, Schuler and Adams, 2010) and this is also true if the exercise is undertaken in gestation, where pregnancy-induced increases in BP and the loss of parasympathetic tone were prevented (Stutzman *et al.*, 2010). A randomised control trial demonstrated that women who exercised during pregnancy from 12 weeks gestation, had improved aerobic fitness and muscle strength by 30-32 weeks of gestation (Price, Amini and Kappeler, 2012). The American College of Obstetrics and Gynecology (ACOG) released physical activity guidelines for pregnant women, with the recommendation that women with uncomplicated pregnancies engage in ≥20–30 minutes/day of exercise on most days/week (ACOG Committee opinion., 2015). The National Institute for Health and Care Excellence (NICE) guidelines similarly encourage continuing or starting moderate exercise during pregnancy (NICE, 2008)

Exercise has been shown to reduce the risk of obstetric complications such as miscarriage (Latka, Kline and Hatch, 1999), GDM (Oken *et al.*, 2007), and pre-eclampsia (Marcoux, Brisson and Fabia, 1989; Martin and Brunner Huber, 2010). It has also been shown to have a

beneficial impact on delivery by reducing both the risk of caesarean delivery (Melzer *et al.*, 2010) and premature delivery (Evenson *et al.*, 2002; Magro-Malosso *et al.*, 2017) as well as promoting a faster recovery postpartum (Price, Amini and Kappeler, 2012). Maternal and fetal health also benefited from exercise in these studies, with an improvement in maternal cardiovascular health through a decreased resting heart rate (HR) and BP (Lynch *et al.*, 2003), improved fetal growth (Clapp *et al.*, 2000), and lower fetal HR (May *et al.*, 2010). The effect on postnatal health has been explored in some studies with activity during pregnancy related to improved neurodevelopment at 12 months (Domingues *et al.*, 2014) and a reduction in overall chronic disease risk (Pivarnik *et al.*, 2006; Moyer, Reoyo and May, 2016).

Meta-analyses of randomized control trials showed that a supervised physical exercise program of low to moderate intensity that includes an aerobic component was able to reduce the risk of GDM by 28-31% and lower GWG by 1.1kg (Sanabria-Martínez *et al.*, 2015). If the exercise was continued throughout pregnancy this reduction in risk increased to 36% (Sanabria-Martínez *et al.*, 2015).

Exercise as an intervention in an overweight/obese pregnancy

Overweight/obese women are two times more likely to exceed the guidelines set for appropriate GWG, and excessive GWG is associated with negative obstetric outcomes (Cedergren, 2006). A study of supervised exercise sessions in overweight/obese pregnant women, called Exercise Training in Pregnancy (ETIP) trial, reduced incidence of GDM and lowered SBP (not diastolic pressure), but failed to reduce GWG (Garnæs *et al.*, 2016). A behavioural intervention may be required to impact GWG, this was demonstrated in the UK Pregnancies Better Eating and Activity Trial (UPBEAT), where GWG was reduced (Poston *et al.*, 2015)

A supervised cycling exercise program was carried out in a randomized control trial of overweight/obese Chinese women. Women in the intervention group had lower incidence of GDM (with reduced insulin resistance at 25 weeks gestation), and less GWG by 25 weeks (Wang *et al.*, 2017). An exercise-only intervention in overweight/obese women improved fitness but did not impact birthweight or maternal/fetal clinical outcomes. Compliance to the protocol was low and variable with only 35% of sessions completed, which decreased as pregnancy progressed (adherence monitored by HR monitors). Increased compliance correlated with improved postnatal adiposity (decreased total fat mass and BMI) of the mothers (Seneviratne *et al.*, 2016).

It may be too premature to say these studies had a disappointing impact on the chosen primary maternal outcomes, as it may have encouraged a change in lifestyle that causes the mother to maintain a healthier BMI for her next pregnancy, and will have also lowered her own risk of cardiometabolic disease. Equally, the exercise may have minimized the adverse "programming factors" (not measured/detected in the study) which will have a positive impact on the baby and their future health. There are very few studies carried out in humans that follow up the mother and baby in order to study the long-term effects. When a study does undertake long-term follow up the results of this will not be elucidated for many years to come. Maternal obesity was associated with a 35% increased risk of premature all-cause offspring mortality by the age of 50 years, therefore even a modest reduction in this risk would have major health and economic benefits for society (Reynolds *et al.*, 2013). Exercise and/or lifestyle intervention studies have had conflicting impacts on maternal outcomes, suggesting the type/intensity and compliance of the exercise protocol is important.

Intervention studies in animal models

A maternal exercise intervention has been shown to improve maternal glucose tolerance and prevent hyperinsulinemia in the obese mother both during pregnancy (Fernandez-Twinn *et al.*, 2017) and during lactation (Vega *et al.*, 2016) without altering maternal bodyweight. These benefits are transmitted to the offspring by improving insulin and glucose homeostasis in adult mouse male offspring (Fernandez-Twinn *et al.*, 2017). Equally, offspring metabolism could be improved by altering body composition, for example an increase in lean mass accompanied by a decrease in fat mass is seen in male offspring from a maternal obesity and exercise model in rats (Vega *et al.*, 2016). In another study of voluntary wheel running in HFD fed mothers, changes in glucose tolerance were seen in the early life of male offspring from obese dams exercised during pregnancy, yet alterations in body composition were not present until offspring were 12 months old (Stanford *et al.*, 2015). Offspring from swim-trained mothers (before and during pregnancy) were protected against diet-induced obesity. Adult offspring were switched to a HFD for four months and in that time offspring from trained mothers gained less weight, which was attributed to decreased fat mass gain, and increased energy expenditure during the day (Wasinski *et al.*, 2015).

HFD-fed rats were provided access to a running wheel ten days before pregnancy; maternal exercise appeared to reduce the metabolic risk of the offspring caused by maternal obesity by improving insulin/glucose homeostasis. The impact on the offspring was sex-specific as males

appeared to benefit more from maternal exercise than females (Raipuria, Bahari and Morris, 2015). A suggested mechanism for the amelioration in glucose metabolism is the reduction by maternal exercise in pro-inflammatory signalling in the hypothalamus of mice offspring, which could result in hypothalamic dysfunction (Bae-Gartz *et al.*, 2015).

1.10.2 Combined diet and exercise interventions

A meta-analysis of 36 randomised controlled trials of diet and physical exercise interventions concluded that they were effective in reducing GWG and the likelihood of caesarean delivery (i-WIP Collaborative group, 2017). The LIMIT trial used lifestyle advice intervention that combined dietary and behaviour changes alongside physical activity in pregnant women with a BMI above 25 kg/m^2. This lifestyle intervention lowered the risk of having a baby with a birthweight of more than 4 kg or having respiratory distress syndrome and was associated with a shorter hospital stay (Dodd *et al.*, 2013, 2014). However, the authors showed no clinically relevant or statistical differences in the measured cardiometabolic and inflammatory markers in maternal and infant cord blood (Moran *et al.*, 2017) which is probably why there was no observed effect in clinical pregnancy outcomes (Dodd *et al.*, 2014). Long-term follow up of these offspring has not yet been completed and could still reveal differences and improvements.

A randomized controlled trial called the UPBEAT used a complex behavioural intervention and addressed diet and exercise in obese pregnant women (Poston *et al.*, 2015). The mean BMI of the pregnant women in the study was 36 kg/m^2, with the inclusion criteria stating the BMI was above 30 kg/m^2. Women in the intervention group had lower dietary glycemic load along with other favourable dietary changes (e.g. decreased intake of saturated fat) and increased physical activity. Behaviour of the mother was altered to a degree by the intervention, however this was not enough to improve glucose tolerance. The study did not achieve the set primary outcomes to reduce the incidence of GDM and LGA babies. GWG and the sum of skinfold thickness in the mother were decreased in the intervention group. Neonatal outcomes did not differ between groups (Poston *et al.*, 2015). The intervention also did not alter cord blood metabolic profiles (Patel, Hellmuth *et al.*, 2017), however a follow up in the offspring at six months revealed a reduction in subscapular skin fold thickness in the intervention arm, and causal analysis revealed this change was likely mediated by maternal antenatal diet (Patel, Godfrey *et al.*, 2017).

Animal models should be used to determine the value of a dietary and physical activity interventions and guide future human intervention studies, due to their ability to enforce exercise and control diet completely. However very few animal models of combined behavioural interventions have been carried out. One study in obese rats performed a dietary intervention where one month before mating they were placed on a control diet which was continued throughout pregnancy and lactation. The dietary intervention was able to reverse some of the adverse metabolic effects of maternal obesity on offspring health (Zambrano *et al.*, 2010). Improvements in offspring metabolic health was also demonstrated in a dietary intervention during pregnancy and lactation in obese mouse dams; further indicating its promise at preventing adverse long-term effects on offspring (Janoschek *et al.*, 2016).

1.10.3 Pharmacological Interventions

Insulin sensitizer: Metformin

Metformin has been given to women affected by polycystic ovarian syndrome with no evidence of causing birth defects (Cassina *et al.*, 2014). The Metformin in GDM (MiG) trial recruited women with GDM at 20-23 weeks gestation. Metformin was started at 500 mg once or twice daily and then increased until the glycemic target was met. Women in the Metformin group had improved insulin sensitivity and reduced GWG (Rowan *et al.*, 2008). Hyperglycemia and insulin resistance occurring with obesity (Jeevendra Martyn, Kaneki and Yasuhara, 2018) are thought to be strong influencers of the observed poor maternal and fetal outcomes (Smith *et al.*, 2008). The Metformin in Obese non-diabetic Pregnant women (MOP) double blinded placebo trial was set up to test the effectiveness of Metformin (*vs.* placebo) in obese pregnant women. The median GWG and the incidence of pre-eclampsia were reduced in the Metformin group. There were no differences in reported adverse outcomes, however there was a significant increase in reported side effects causing 18% of women to stop taking Metformin, but despite this the overall adherence to the trial was considered good (Syngelaki *et al.*, 2016).

Increased reporting of side effects was also reported in the Effect of Metformin on maternal and fetal outcome in obese pregnant women (EMPOWaR) trial (another double blinded placebo controlled trial). This study did not show any differences in maternal outcomes or GWG with administration of Metformin, but the discordance between the two studies could be explained by the lower adherence to the metformin dose and decrease in successful recruitment/participation for the study (Chiswick *et al.*, 2015). There was a reduction in

HOMA-IR and fasting glucose at 28 weeks gestation, however this was not present at 36 weeks. Markers for inflammation CRP and IL6 maternal serum concentrations were also lowered in the Metformin group (Chiswick *et al.*, 2015), and this may be beneficial as high levels have been associated with adverse pregnancy outcomes (Xu *et al.*, 2014).

The MiG trial has followed up the offspring to two years old and demonstrated increased measures of skinfold thickness with no overall change in total fat when measured by DEXA (Rowan *et al.*, 2011). These children have increased subcutaneous fat and further follow up is required to determine if changes in their fat distribution has any consequences to their metabolic health later in life.

Metformin administration in animal models of maternal obesity revealed positive impacts such as decreased inflammatory markers in fetal serum (Desai *et al.*, 2013). If offspring were weaned onto a HFD, *in utero* metformin-exposed offspring gained less weight and adiposity, additionally the HFD-triggered impaired glucose tolerance was prevented (Salomäki *et al.*, 2014). Prenatal metformin administration prevents hypertension in HFD fed offspring exposed to a maternal high fructose diet during pregnancy (Tain *et al.*, 2018).

Antioxidants

Oxidative stress is a well-recognized mechanism underlying developmental programming (Thompson and Al-Hasan, 2012). Elevated levels of oxidative stress have been shown in the placenta of obese women (Oliva *et al.*, 2012) suggesting antioxidant treatment could be a relatively unexplored intervention. Oxidative stress can cause DNA damage, lead to cell death, and ultimately accelerate cellular ageing (Tarry-Adkins and Ozanne, 2017). Antioxidants protect against reactive oxidative species and are considered a therapeutic agent to prevent the subsequent damage. Oxidative stress has been shown to be present in pre-eclampsia (Burton *et al.*, 2009) which often co-exists with other adverse pregnancy outcomes such as IUGR, placental previa, and fetal death (Turpin *et al.*, 2015). Two large randomised controlled trials demonstrated a lack of an effect of an antioxidant regimen (Vitamin C + E) in preventing pre-eclampsia in either high-risk (Villar *et al.*, 2009) or low-risk pregnancies (Roberts *et al.*, 2010). The unexpected results of these studies could imply that there is no causative role of oxidative stress, despite it being present in the aetiology of pre-eclampsia, or that the supplementation regimen or the antioxidant used is inappropriate.

A high level of oxidative stress and inflammation has also been shown in the hearts of the offspring using a sheep model of maternal overnutrition. These hearts demonstrated impaired *ex vivo* cardiac function in response to high workload (Wang *et al.*, 2010). An antioxidant (Quercetin) was used as an intervention in HFD-fed mouse dams during pregnancy. This supplementation prevented hyperinsulinemia, hypertension, and HFD-induced weight gain at 12 months of age in the offspring (Liang, Oest and Prater, 2009).

1.11 Thesis Aims and hypotheses

The aims of this thesis were to develop an intervention that can override the unfavourable maternal legacy of obesity. If the increased risk, associated with maternal obesity, to the offspring is not addressed then it will strongly impact the disease risk of the next generation, causing an insurmountable healthcare burden across the world. It is therefore valuable to implement an intervention during development and before birth to improve that individual's cardiovascular health. This should bridge the gap in current successful prevention strategies by employing a strategy that starts before the pathogenic progression of disease.

Interventions during pregnancy could create a healthy environment *in utero* during a critical time during organ development. Women who adopt healthy lifestyle behaviours during pregnancy can positively impact postnatal health and decrease their child's future risk of developing NCDs, such as obesity, T2D, and CVD. At the same time, mothers improve their own future health prospects, therefore such an approach targets more than one individual through a single intervention.

Studying interventions that can normalise a defined maternal parameter can also help identify which critical programming factor is driving the effects in the offspring. Since obesity is a multifactorial disease, which involves many parameters that are known to affect fetal programming (e.g. insulin, glucose, cholesterol and free fatty acids), identifying key factors is important in the understanding behind fetal programming.

The hypothesis of this thesis is that a maternal exercise intervention during pregnancy will prevent programmed offspring poor cardiovascular health.

This hypothesis was investigated through 4 specific objectives:

1. To investigate the effect of an exercise intervention during pregnancy on the metabolic phenotype of obese mouse dams.

2. To determine whether a maternal exercise intervention during an obese pregnancy improves the cardiovascular health of male offspring from obese dams.

3. To assess, for the first time in this model, the impact of maternal obesity on the cardiovascular health of female offspring and then to determine the effect of the maternal exercise intervention.

4. To study the immediate consequences of maternal obesity on the fetal heart and to then identify the impact of maternal exercise intervention.

2. General Methods

2.1 Diet-induced obesity animal model

All animal studies were conducted according to the UK Home Office Animals (Scientific Procedures) Act 1986 and following ethical review by the University of Cambridge Animal Welfare and Ethical Review Board (AWERB). This thesis capitalised on an established maternal diet induced-obesity model using C57BL/6J mice as previously described (Samuelsson *et al.*, 2008; Fernandez-Twinn *et al.*, 2012). From four weeks of age the diets were introduced *ad libitum*; females were either fed a standard control RM1 diet (Special Dietary Services) or a highly palatable energy-rich obesogenic diet (45% Atwater fuel energy (AFE) fat, custom made diet 824053, Special Dietary Services) supplemented with sweetened condensed milk (Nestlé) and fortified with mineral and vitamin mix (AIN93G, Special dietary Services) (diet composition in Tables 2.1+2.2). After three weeks on the respective diets, females were mated for a first pregnancy to ensure they were proven breeders and establish good maternal care. Time-Domain Nuclear Magnetic Resonance (TD-NMR) (Bruker minispec LF series, Bruker Optik GmbH, Germany) was used to determine body composition. Obesogenic diet-fed females were maintained on their diet until they achieved a fat mass ≥ 10 g, when they were then considered obese and entered into the study for the experimental pregnancy. In keeping with this, control females who had a fat mass above 5 g were excluded from the study. This cut off was developed to ensure the reproducibility in the effects of maternal obesity. These two groups of females were rested for one week (to match for the training week undertaken by the obese-exercised group) before mating and weekly TD-NMR measurements were taken before mating and throughout pregnancy. The experimental schematic is shown in Figure 2.1. Females were mated with previously LAD1-fed males (diets were matched to the incoming female during the period of mating), they were left to mate for a maximum of five days and were removed upon a positive vaginal plug. Females were maintained on their respective experimental diets throughout pregnancy and lactation (Schematic shown in Figure 2.1). By the time females were mated for their second pregnancy they had been on the diet for approximately 15 weeks and were approximately 18 weeks old.

TABLE 2.1: Composition of maternal diets. Metabolisable energy for each diet component is shown in brackets. Composition is broken down by % (w/w).

	Control dams	Obese dams	
	RM1 pellets (10.74 kJ/g)	HFD pellets (28.43 kJ/g)	Condensed milk (13.7 kJ/g)
Simple sugars (w/w)	7%	10%	55%
Fat (w/w)	3%	20%	8%
Protein (w/w)	15%	23%	8%
Polysaccharide (w/w)	50%	28%	-

TABLE 2.2: Detailed composition of custom-made high fat diet pellet.

	% (w/w)
Casein	26.5
Cellulose	6.2
Choline bitartrate	0.3
L-cysteine	0.4
Lard	18
Mineral mix	4.3
Rice starch	28.3
Soya oil	4.3
Sucrose	10.5
Vitamin mix	1.2

2.2 Maternal exercise intervention

A third group of obese females (≥ 10 g fat mass) were randomly allocated to the intervention group and were trained to run on a treadmill. The protocol was started when obesogenic diet-fed mice reached 10 g fat mass as measured by TD-NMR (one week before mating). For the first five days treadmill training was carried out, the speed and duration of treadmill running gradually increased incrementally to allow for familiarisation, so that by day five the full protocol was achieved. The full program was then carried out five days a week (Mon-Fri) for

20 minutes/day with a weekend of rest and this was continued from mating up to and including gestational day 17 (protocol shown in Table 2.3). These mice are referred to as obese-exercised (Ob-Ex) (schematic shown in Figure 2.1).

TABLE 2.3: Exercise protocol with speed and durations. By training day 5, females were completing the full program and this was continued until gestational day 17.

Speed (m/min)	*Duration at set speed (min)*				
	Training day 1	*Training day 2*	*Training day 3*	*Training day 4*	*Training day 5 (Full Program)*
5	1	1	1	0.5	0.5
7.5	13	18	1	1.5	1.5
10	-	-	16	3	1
12.5	-	-	-	10	14
10	-	-	-	3	1
7.5	-	-	1	1.5	1.5
5	1	1	1	0.5	0.5

Litter size was standardized to six (in all groups) at postnatal day 2 (PND2) to ensure standardized milk supply and maternal attention. The *n* number for all experiments corresponds to the number of independent litters to eliminate confounding within-litter effects and only one male or female per litter was used for each outcome measure. The offspring were weaned at three weeks of age onto standard laboratory chow (RM1) and fed *ad libitum*. TD-NMR measurements were undertaken weekly in offspring from four to eight weeks of age. At eight weeks of age, mice were fasted overnight (16 hours). Tail blood glucose was measured (AlphaTRAK 2, Zoetis, USA) before the mice were then killed by a rising CO_2 concentration. At *post mortem*, blood was collected by cardiac puncture before tissue was collected, weighed and frozen at -80 ◦C.

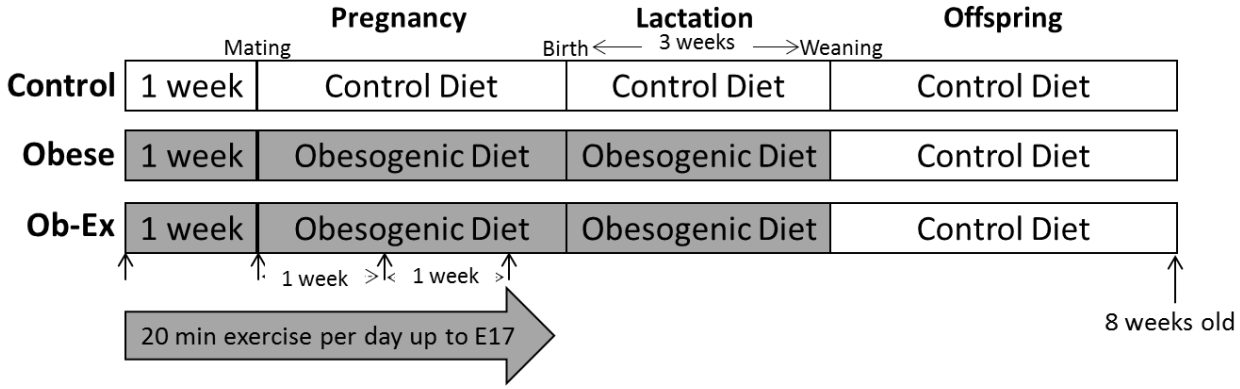


FIGURE 2.1: Schematic of study design with exercise protocol of obese females. Small arrows below represent TD-NMR time points. Treadmill running was started 1 week before mating. Offspring were weaned at 21 days old.

2.3 Serum analysis

Blood was left to clot at room temperature for at least 30 minutes before it was centrifuged at 900 g for five minutes, the supernatant serum was collected and snap frozen (-80°C). Serum metabolites were measured by the MRC-MDU Mouse Biochemistry Laboratory (Addenbrooke's Hospital, Cambridge, UK). Serum Leptin and Insulin were measured by Ultrasensitive Mouse Leptin ELISA kit (Crystal Chem INC, 90030) and Ultrasensitive Mouse Insulin ELISA kit (Crystal Chem INC, 90080) respectively, according to manufacturer's instructions.

2.4 Non-invasive tail cuff plethysmography

BP was measured non-invasively by restraint tail cuff plethysmography using a BP-2000 Series II machine (Visitech systems, USA) in offspring at seven weeks of age. SBP can be determined by monitoring tail vessel dilation through the scattering of red light as the occlusion cuff inflates. Prior to being measured, animals had been handled frequently (due to weighing and TD-NMR measurements). This was important as it minimised stress the animal experienced during the BP measurement. Over three consecutive days there were two training days to enable familiarisation of the animal to restraint. On the third consecutive day, five pre-measurements were taken by the machine but were not used for the analysis. Following this, 10 measurements of SBP and pulse rate were recorded for each session. Only measurements recorded on the final (third) day were used for analysis to reduce variability in the results. On the third day, when the variability of the data was the lowest, all animals included in analysis had a co-efficient of variation of 10% or less. Measurements were taken

at the same time each day (16:00) to account for the natural circadian variation of BP over a 24 hour period (Staessen *et al.*, 1992).

2.5 *In vivo* echocardiography

Left ventricular (LV) transthoracic echocardiography was carried out using the Vevo770 system and RC707B 30 MHz cardiac transducer (FUJIFILM, Visual Sonics, Canada). Mice were anaesthetised via inhalation of 2% isoflurane in O_2 and anaesthesia was maintained through the use of a nose cone at 1.5% isoflurane. Mice were placed in a supine position on a heated platform and their limbs were taped over metal electrocardiogram (ECG) pads allowing for real time ECG monitoring. A rectal probe was used to monitor body temperature which was kept strictly in an appropriate range. HR was constantly monitored and kept >400 beats per minute (bpm) by adjusting depth of anaesthesia if necessary. To gain an appropriate acoustic window, fur on the animals' thorax was removed through the use of hair removal cream. Pre-warmed ultrasound gel was also utilized. The transducer was positioned to give parasternal long-axis view of the LV and focused at the level of the papillary muscle. In B-mode the aorta was lined up with the apex of the heart. M-mode images and an EKV^{TM} were captured in this view (Figure 2.2). Measurements were carried out on the M-mode software using Vevo770 software. Cardiac functional outcomes were calculated using the LV tracing function while lumen and wall dimensions were measured using the in-built measurement platform on M-mode images (for calculations used in software refer to Appendix AP1). This data was collected in partnership with Dr Denise Fernandez-Twinn and Dr Heather Blackmore, with two people there for all measurements as mandated by AWERB.

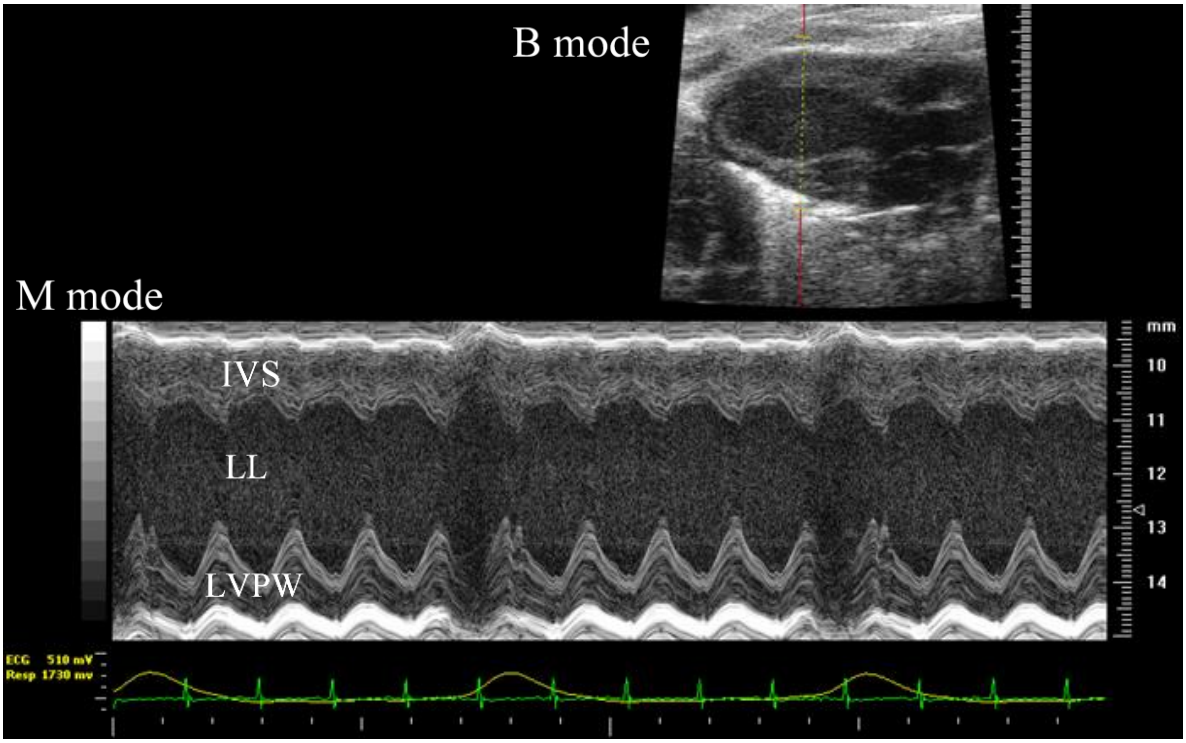


FIGURE 2.2: Representative image showing alignment of heart in B-mode and M-mode. B-mode was aligned in the parasternal long-axis view of the LV. Using the Vevo770 software an overlay was placed on the B-mode image equidistant between the apex and the start of the aorta opening. M-mode images were then captured from this position in the ventricle. IVS- Interventricular septum, LL- Left lumen and LVPW- LV posterior wall are labelled on the M-mode image.

2.6 RNA extraction and quantification

Snap-frozen ventricular heart tissue stored at -80ºC was powdered over dry ice with a pre-chilled pestle and mortar. 25 mg aliquots of powdered tissue were weighed for RNA extraction using a column based miRNeasy mini kit (Qiagen, Germany) following manufacturer instructions. Powdered tissue was lysed with a TissueRuptor homogeniser (Qiagen, Germany) in Qiazol. Chloroform was added and samples were incubated at room temperature. After a centrifugation step (12,000 g at 4ºC for 15 minutes) the upper aqueous layer was removed and placed in a fresh tube. 1.5 volumes of 100% ethanol was added and mixed. This was then pipetted into the columns provided and centrifuged for 15 seconds (> 8,000 g at room temperature) to pull solution through column. A DNase digest was then performed (RNase-Free DNase set, Qiagen, Germany) to remove any contaminant DNA. The final RNA product was eluted in 30 µl of RNase free water.

RNA concentration (ng/µl) was measured by Nanodrop (ThermoScientific, UK). Ratios of absorbances measured at 280 nm, 260 nm, 230 nm were measured alongside concentration to

assess the purity of the RNA. The 260/230 nm ratio indicates contamination of the sample with protein or phenols, a ratio of 2 is considered to be pure RNA. 280/260nm ratio indicates the presence of salts therefore an ideal ratio is between 1.8-2.2. The integrity of the extracted RNA was assessed by identifying the 28S and 18S ribosomal RNA bands following electrophoresis using a 1% agarose gel. The RNA sample was diluted to 100 ng/µl and the concentration was checked again using the Nanodrop (ThermoScientific, UK) and adjusted if necessary.

2.6 Quantitative PCR

10 µl of RNA (1 µg) was used for the reverse transcriptase (RT) reaction using a high capacity cDNA RT kit (ABI, USA), which was then added to 10 µl/reaction of mastermix (prepared as shown in Table 2.4). The RT reaction was performed using a Veriti 96 well thermal cycler (ABI, USA) as per manufacturer's instructions (protocol shown in Figure 2.3).

TABLE 2.4: Volumes of RT reaction for a single reaction.

Provided in RT kit	*1x reaction (µl)*
10 RT buffer	2.0
25 dNTP mix	0.8
10x RT primers	2.0
Multiscribe RT	1.0
RNase inhibitor	1.0
Nuclease free H_20	3.2

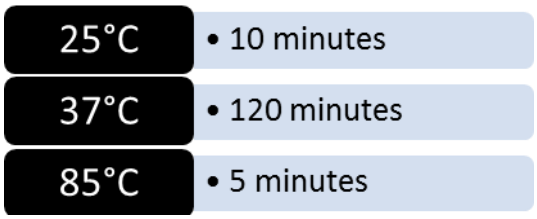


FIGURE 2.3: Program of the thermocycler conditions.

cDNA was diluted to a suitable dilution factor and stored at -20°C. 5 µl of each sample was added together to create a pooled sample. The 100% pooled sample was used to generate a pooled standard curve by serial diluting 1:2 to give six standards. This was achieved by adding nuclease free water to an equal volume of sample. Samples were then diluted 1:10 to

ensure that they lie in the middle of the newly generated pooled standard curve. The linear equation of the line of best fit was then used to calculate relative gene expression.

2.6.1 Validation of primers

Where possible, previously validated primers were used and when this was not possible primers were designed to be intron spanning using the Ensembl database. Target specificity was assessed using Primer-blast (National Center for Biotechnology Information, USA) and those with unintended targets were redesigned. Primers were also assessed for their 3' self-complementarity, with primers of the lowest value chosen. Running a melt curve was used to assess target specificity in validated primers. If there was more than one peak, the primer was not specific to the target and was also binding to an off-target region, such a primer would not be used and would be redesigned. Primer sequences are shown in the Chapter 4 and 5.

Each primer was tested individually and in duplicate in samples and pooled standards using SYBRgreen technology. For each reaction the components were as follows: 5 µl cDNA dilution, 6 µl SYBR green mastermix and 1 µl Primer (1.6 µm). Real time PCR using SYBRgreen PCR mastermix (ABI, USA) was carried out using a 384 well 7900HT fast real-time PCR system. Housekeepers were used to normalise gene expression and are defined in methods of the individual chapters.

2.7 Protein extraction

Powdered ventricular tissue (25 mg) was homogenised on ice in lysis buffer [50 mmol/L HEPES, pH 8; 150 mmol/L sodium chloride; 1% Triton X100; 1 mmol/L sodium orthovanadate; 30 mmol/L sodium fluoride; 10 mmol/L sodium pyrophosphate; 10 mmol/L EDTA with a protease inhibitor cocktail (set III), Calbiochem Novabiochem Biosciences, UK]. Homogenates were placed on a shaker at 4°C for 15 minutes before centrifuging for five minutes at 15700 g and removing supernatant to a new tube. Total protein concentration of homogenates was measured in duplicate by copper-bicinchoninic assay (Sigma-Aldrich, UK) in a 96 well plate. A standard curve was generated using a series of known protein concentration of bovine serum albumin (BSA). Absorbance at 562 nm were measured in a Tecan M1000 Pro plate reader (Tecan, Switzerland). The lysates were then diluted in lysis buffer to 1 mg/µl. 40 µl of 5x Sodium dodecyl sulphate (SDS) loading buffer (25mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 10% (w/v) SDS, 500mM Dithiothreitol (DTT),

Bromophenol blue) was added to lysate dilutions (200 µl) in a 1:5 dilution. Protein lysates were stored at -20°C.

2.8 Western blotting

All buffers and compositions detailed in Table 2.5.

2.8.1 SDS PAGE

Resolving acrylamide gels (8-15%) were prepared depending on size of protein with the stacking gel containing 5% acrylamide. For Chapter 4, gels were set between glass plates with 3 mm spacers and rubber tubing. For Chapter 5, gels were set between 1.5 mm spacers using the SE 600 Ruby Standard Dual Cooled Vertical Unit (GE healthcare, USA) system. An appropriate comb was used to set a maximum of 24 wells. After gels were set, they were placed in an electrophoresis tank with 1x SDS-PAGE running buffer. Protein samples were heated to 95ºC for five minutes before loading. 15 µg of protein/sample and 10 µl of protein ladder (10-250 kDa, PageRuler pre-stained protein markers, ThermoScientific, UK) were loaded into a polyacrylamide gel of appropriate percentage and separated electrophoretically in running buffer 40 V overnight (~16 hours) until the gel front reached the bottom of the gel and the band on interest was in the middle of the resolving gel.

2.8.2 Coomassie stain: equal protein loading

To confirm equal protein loading across all samples the first gel of the study was stained by Bio-safe Coomassie Brilliant blue stain (Bio-Rad Laboratories, UK). The gel was prefixed (50% Methanol, 10% Acetic acid and 40% H_2O) for 30 minutes before the Coomassie stain was added for 2-4 hours at room temperature or until the gel was uniformly stained blue. A destaining solution was used until background was completely clear. The gel was imaged using Bio-Rad ChemiDoc Imager (USA), corresponding images are shown in Chapters 4 and 5.

2.8.3 Semi-dry transfer

Proteins separated by SDS PAGE were then transferred onto a polyvinyl difluoride (PVDF) membrane (Immobilon-P; Millipore, USA) using a TE70XP Semi-Dry Transfer Unit. The membrane was first activated by immersing in 100% methanol for one minute, dH_2O for one minute and then transfer buffer for five minutes. Blotting paper (195 g/m^2) was cut to gel size and pre-soaked in transfer buffer, three pieces of blotting paper were laid down onto the damp transfer surface, the pre-cut activated PVDF membrane was stacked on top followed by the

gel and a further three layers of pre-soaked blotting paper. Care was taken to ensure no bubbles are introduced in the stack that would impede effective transfer. Transfer was set at 200 mA for one hour 30 minutes. Transfer efficiency was confirmed with Ponceau S staining.

2.8.4 Blocking and antibody incubations

Membranes were blocked with 5% skimmed milk protein (Marvel, Premier Foods, UK) in 1x Tris-buffered Saline (TBS) - 0.1% Tween 20 for at least one hour at room temperature. After which they were incubated in primary antibody (in 5% BSA (TBS-Tween) buffer) at 4ºC overnight. The following day they were washed in 1x TBS - 0.1% Tween 20 for 15 minutes, changing the solution every five minutes, before incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody for one hour (1:10,000 for all antibodies) (Jackson ImmunoResearch, Stratech, UK). A second wash step of 15 minutes in 1x TBS - 0.1% Tween 20 was undertaken after secondary antibody incubation. Antibody binding was detected using Luminata Forte Western HRP substrate (Millipore, USA) and chemiluminescent bands were quantified directly with Bio-Rad ChemiDoc Imager (BioRad, USA).

TABLE 2.5: Buffer components for western blotting.

Buffer	Components of buffer
Running buffer	25 mM Tris-Base 200 mM Glycine 0.1% SDS
Transfer buffer	50 mM Tris-Base 40 mM Glycine 0.15% (w/v) SDS 20% (v/v) Methanol
Resolving gel % acrylamide in the gel was chosen based on size of protein of interest; to allow for optimum separation of bands.	8-12% Acrylamide (40%) 0.05% Bisacrylamide (2%) 37.5 mM Tris-HCl 1% (w/v) 20% SDS 0.05% Tetramethylethylenediamine (TEMED) 0.005% Ammonium persulphate (APS) (10%)
Stacking gel	5% Acrylamide (40%) 0.05% Bisacrylamide (2%) 12.5 mM Tris-HCl 1% (w/v) 20% SDS 0.05% TEMED 0.005% APS (10%)
Tris-Buffered Saline ((TBS) (10x) pH 7.6))	50 mM Tris-Base 150 mM Sodium chloride
TBS with 0.1% Tween (TBS/T)	1x TBS 0.1% Tween-20
Blocking buffer	1x TBS, 0.1% Tween-20 5% (w/v) non-fat dehydrated milk
Antibody dilution buffer	1x TBS, 0.1% Tween-20 5% (w/v) bovine serum albumin (BSA)
1M Tris-HCl (pH 6.8)	121.14 g Tris-Base in 1 litre dH_20
3M Tris-HCl (pH 8.8)	363.42 g Tris-Base in 1 litre dH_20

2.9 Statistical Analysis

All data analysis was carried out using Prism 6.0 (GraphPad, USA). Prior to statistical analysis, data were tested for statistical outliers using the Grubbs test (GraphPad, USA). Statistical outliers ($p< 0.05$) were removed from the analysis. Data was analysed by one-way analysis of variance (ANOVA) to enable the comparison of three experimental groups and assess the effect of maternal lifestyle. Maternal lifestyle was used to describe the effect of maternal obesity with and without the exercise intervention. When the same measurement was carried out at multiple time points (e.g. TD-NMR) a two-way ANOVA was used to assess the effect of maternal lifestyle and time. Where possible, a repeated measures correction was utilised in this analysis.

Post-hoc testing was carried out using the Bonferroni method. For all data, the mean and standard error of the mean (SEM) are presented and $p< 0.05$ was considered statistically significant. Sample sizes (n) are reported within each figure legend. For offspring data, the sample size represents the number of independent litters per experimental group, this avoids litter bias as only one male or female was used per litter.

3. Assessment of the maternal phenotype in a mouse model of diet-induced obesity

3.1 Introduction

3.1.1 Why study maternal obesity?

As highlighted in Section 1.2, the world is experiencing an obesity epidemic that is affecting women of childbearing age. There is a large body of evidence supporting the impact of adverse early life exposures *in utero* and during early postnatal life on the programming of disease in adulthood. Subsequently, the rising levels of maternal obesity has the capacity to negatively impact on the health of future generations.

Why generate an animal model of maternal obesity?

The choice of the animal model is often a balance between the cost and practicality of the research, and the translatability to humans. Although NHPs are closest to human development their long gestation time leads to high research costs. In addition, their long lifespan makes longitudinal studies into adulthood a challenge. Ovine models can be used as, like humans, they usually have a singleton pregnancy, the lamb is born at a similar weight to a human baby and they are a good model for studying fetal physiology as they allow fetal catheterisation. Rodent models have been used extensively due to the short gestation time and relatively low costs compared to larger animals.

In general, animal models of maternal overnutrition lead to outcomes in offspring that are similar to those seen in epidemiological studies. This is discussed in more detail in Section 1.9. The ability to remove confounding factors and identify causal relationships has highlighted that subtle environmental changes can have important consequences for offspring health. Animal models are also able to uncouple maternal characteristics that would usually be interconnected in humans. This has helped in the identification of "programming factors" associated with maternal obesity that could mediate detrimental effects in the offspring.

3.1.2 Contributing factors to adverse development

During pregnancy there is an increased risk of hypertension, metabolic disturbances of nutrient metabolism, and inflammation; this is exaggerated when accompanied with obesity (Catalano, 2010). Any alterations from the normal responses to pregnancy that occurs in a

suboptimal intrauterine environment could represent "programming factors" in the mother that mediate the offspring outcomes.

Insulin resistance

Pregnancy is characterised by significant changes in physiology, endocrinology and metabolism that enable the mother to sustain fetal requirements for both oxygen and nutrients. This is achieved by creating a pseudo-diabetogenic state that results in progressive insulin resistance. By late pregnancy, insulin sensitivity is reduced by 50% in normal weight women compared to the non-pregnant state (Catalano *et al.*, 1991). When the normal process of pregnancy-induced insulin resistance is exaggerated, and there are accompanying metabolic abnormalities in glucose metabolism, GDM can develop (Mottola and Artal, 2016). This is often the case in maternal obesity as obesity is usually accompanied by a pre-existing state of insulin resistance. GDM pregnancies are associated with increased obstetric complications (Section 1.5.1) and fetal macrosomia (Yessoufou and Moutairou, 2011).

Maternal leptin

Overweight or obese women have higher circulating levels of serum leptin (Misra, Straughen and Trudeau, 2013). The main determinant of serum leptin levels is thought to be adiposity (and BMI) and this is also true in the pregnant state (Misra and Trudeau, 2011). However this dogma becomes more complex during pregnancy, as leptin nearly doubles over the course of pregnancy with leptin production by the placenta thought to play a role in this substantial increase (Hauguel-de Mouzon, Lepercq and Catalano, 2006). This has implications for the fetus; maternal leptin correlates with cord blood leptin levels taken immediately after delivery and this is associated with altered anthropometric characteristics (Marino-Ortega *et al.*, 2015). A postnatal leptin surge that occurs in the postnatal period in rodents is critically important for organogenesis, especially within the brain (Attig *et al.*, 2011). Leptin remains elevated in a rat model of maternal obesity during the postnatal period, evidenced by an exaggerated leptin surge and an increase in leptin found in the pup's stomach throughout lactation (as a proxy measure of leptin in the mother's milk) (Kirk *et al.*, 2009). This is thought to alter sympathetic tone through melanocortin signalling in the hypothalamus (Samuelsson *et al.*, 2016). The subsequent sympathetic hyper-stimulation in the developing renal-cardiovascular system could lead to hypertension and cardiovascular dysfunction in adulthood (Briffa *et al.*, 2015).

Oxidative stress

An imbalance between reactive oxygen species (ROS) production and the cellular antioxidant defense system leads to oxidative stress. Obesity causes an increase in oxidative stress and inflammation, the resultant oxidative damage may contribute to the onset and development of many pathologies (Furukawa *et al.*, 2004). In particular, maternal obesity results in dysregulation of the redox balance in the maternal-placental-fetal unit and this induces placental and fetal oxidative stress despite the high placental antioxidant defense (Malti *et al.*, 2014).

Oxidative stress is associated with pregnancy complications such as pre-eclampsia (Atamer *et al.*, 2005) and IUGR (Hracsko *et al.*, 2008), which could contribute to the postnatal consequences for the offspring. Animal studies are starting to provide evidence that oxidative stress is important in the programming of CVD. Offspring of obese mouse dams have enhanced lipid peroxidation and impaired antioxidant capacity in their hearts, suggesting oxidative stress is a potential driving force for the development of CVD in the offspring (Fernandez-Twinn *et al.*, 2012). Low protein diet-exposed rat offspring exhibited elevated arterial BP alongside exaggerated vasoconstrictor response to Angiotensin II; these alterations were prevented by the prenatal administration of the antioxidant, Lazaroid (Cambonie *et al.*, 2006).

3.1.3 Why exercise as an intervention during pregnancy?

Exercise could be an effective intervention as it is able to target some of the adverse programming factors outlined in Section 3.1.2. There are a growing number of pregnancy intervention studies that utilise exercise (reviewed in Section 1.10.1) to improve maternal and neonatal outcomes. Both NICE and ACOG recommend exercise during pregnancy for all women with uncomplicated pregnancies, as there is no evidence of harm and indeed evidence suggests improvements in pregnancy outcomes (NICE, 2008; ACOG Committee opinion., 2015). Exercise, therefore, presents an attractive candidate for an intervention during a suboptimal pregnancy, such as maternal obesity.

Ability to target insulin resistance

In non-pregnant humans, a short bout of exercise has been shown to result in increased insulin sensitivity that persists for several hours after exercise (Maarbjerg, Sylow and Richter, 2011). A randomised control trial was undertaken in healthy pregnant women to assess the effect of a triweekly exercise protocol on glucose tolerance. Blood glucose level one hour after an oral

glucose challenge (50 g) was lower in the exercised group suggesting improved glucose tolerance (Barakat *et al.*, 2012). Exercise can also be used to regulate glucose tolerance in women who are at high risk of developing GDM. Examples of risks include family history of diabetes, hormonal disorders such as polycystic ovarian syndrome, overweight/obesity and excessive GWG (Pons *et al.*, 2015). Glycemic control is considered the cornerstone of GDM treatment, which can be achieved not only by control of diet (Hoffert Gilmartin, Ural and Repke, 2008) but could also be addressed by exercise. The development of GDM is an important complication to address as it has significant negative obstetric outcomes such as macrosomia, caesarean delivery, pre-eclampsia and other pregnancy induced-hypertension disorders; many of which will have important influences on the fetus (Ehrenberg *et al.*, 2004; Yessoufou and Moutairou, 2011).

Ability to attenuate oxidative stress

Oxidative stress associated with obesity during pregnancy may be a contributing factor in the induction of programmed phenotypes (see Section 3.1.2). Exercise is able to attenuate oxidative stress and inflammation (Durrant *et al.*, 2009; Peppler *et al.*, 2016). Voluntary running in lean pregnant rats increases placental efficiency and antioxidant concentrations, which could attenuate oxidative stress (Gilbert *et al.*, 2012). Wheel running in obese pregnant mice lowers markers of oxygen and nitrogen stress such as serum malondialdehyde and liver superoxide dismutase (Vega *et al.*, 2016). Using the same model of diet-induced obesity used for this thesis, maternal obesity was associated with placental hypoxia and this was attenuated by a maternal exercise intervention (Fernandez-Twinn *et al.*, 2017). Oxidative stress is an important mediator and being able to target this potentially harmful programming factor could, therefore, have significant implications for the offspring.

3.1.4 Aims of the chapter

The first aim of this chapter was to determine the impact of an obesogenic diet on maternal bodyweight and body composition during pregnancy and to then assess the effects of a maternal exercise intervention. The second aim was to characterise how neonatal growth profiles were altered with maternal obesity and if this was modified by maternal exercise. The final aim was to assess whether the impaired glucose tolerance and hyperinsulinemia observed in obese dams during pregnancy was still present at the end of lactation.

3.2 Methods

3.2.1 Body composition

The detailed description of the experimental protocol is described in Section 2.1 and 2.2. Maternal body composition (fat and lean mass) was measured weekly by TD-NMR for four weeks starting one week before mating, during mating and for the first two weeks of pregnancy. For Obese and Ob-Ex groups the start of the study was when a minimum fat mass of 10 g fat was reached. The exercise protocol was started a week before mating and this first week was designated the training week. Female mice were placed with stud males for mating for a maximum of five days and then were removed to be singly housed when a vaginal plug was detected. Food intake was measured from the start of pregnancy (day of plug) until the dam gave birth. Dams were singly housed throughout this time. RM1 diet pellet, HFD pellet and condensed milk were weighed weekly to calculate intake. Food intake was not measured during lactation to avoid confounding factors of the pups also consuming the diet and the potential stress it may cause to the dams and pups.

When dams littered, the dam and pups were not disturbed until PND2 when the litter size and sex was noted and the litter was weighed. Litter size was standardised to six pups to ensure equal nutritional access and maternal care. Fraction of male pups was calculated by dividing the number of male pups by the total number of pups in the litter. Whole litter weight was subsequently measured on PND7, PND14 and PND21. The average weight of each pup in the litter was then calculated by dividing the total litter weight by the number of pups in the litter. Fractional growth rate (FGR) was calculated by the following formula:

$$FGR = \frac{(PND21\ weight - PND2\ weight)}{(PND21 - PND2)} / PND2\ weight$$

This gives the bodyweight gained per day during lactation as fraction of the weight on PND2. At three weeks of age mouse offspring were weighed individually, separated by sex and housed as a pair with a same sex littermate. All offspring were weaned onto a control (RM1) diet.

3.2.2 Post weaning intraperitoneal glucose tolerance test (GTT)

On the day of weaning, the dams were fasted overnight beginning at 16:30. Dams were placed individually into a clean cage with access to water. They were fasted for 16 hours and blood drawn from the tail for basal (0 minute) glucose measurements (AlphaTRAK2, Zoetis, USA).

Dams were then injected intraperitoneally (i.p.) with 1 g/kg glucose and further tail blood glucose measurements were made at timed intervals after injection (15 minutes, 30 minutes, 45 minutes, 60 minutes, 90 minutes and 120 minutes). Tail blood was collected at 0 minutes, 15 minutes and 30 minutes time points in haematocrit Na- heparin capillary tubes (Hirschmann- Laborgeräte, Germany). Plasma was isolated by spinning tubes in Haematospin (Hawksley, UK) for four minutes and plasma insulin was measured using a Mouse Insulin ELISA (CrystalChem, USA) following manufacturer's instructions. Area under curve was calculated by summation of trapezoids (Prism, GraphPad, USA). Using the matched plasma insulin and glucose values HOMA-IR was calculated using the HOMA calculator (Diabetes Trial Unit, University of Oxford available here: https://www.dtu.ox.ac.uk/homacalculator/).

3.2.3 Statistical analysis

When the same measurement was taken at multiple time points (e.g. TD-NMR, food intake, lactation bodyweight and glucose curve) data was analysed by two-way ANOVA with repeated measures correction and a Bonferroni post-hoc test. The three groups were compared by one-way ANOVA and a post-hoc Bonferroni test. All data is presented as mean ± SEM.

3.3 Results

3.3.1 Maternal body composition and food intake

There was a significant effect of maternal lifestyle ($p< 0.001$) and time ($p< 0.001$) on dam bodyweight from one week before and during mating and pregnancy (Figure 3.1A). Post-hoc testing demonstrated that obesogenic diet-fed dams had increased bodyweight throughout mating and pregnancy compared to control dams. There were no differences in bodyweight due to the exercise intervention (Obese vs. Ob-Ex dams) (Figure 3.1A).

Over mating and pregnancy there was a significant effect of maternal lifestyle ($p< 0.001$) and time ($p< 0.001$) on dam fat mass, with post-hoc testing showing increased fat mass in obesogenic diet-fed dams at every time point measured compared to controls. This is not surprising as at the start of the study the fat mass was kept below 5 g for the control diet-fed dams and over 10 g for the obesogenic diet-fed dams. Maternal exercise did not alter fat mass over the intervention period. There was a significant interaction between the week of TD-NMR measurement and maternal lifestyle, this means that the week of the TD-NMR measurements had a different effect on obesogenic diet-fed dams when compared to control

dams. This suggests that obesogenic diet feeding not only increases fat mass of the dam over mating and pregnancy but also that they gain more fat mass over this period (Figure 3.1B).

There was a significant effect of time ($p< 0.001$) and maternal lifestyle ($p< 0.05$) in the lean mass of dams. Post-hoc testing detected differences in the second week of pregnancy; the obesogenic diet-fed dams had decreased lean mass compared to the control dams. There was also a significant interaction, suggesting the effect of the week of TD-NMR measurement on dam lean mass is different between the groups. This could highlight the smaller gain in lean mass in obesogenic diet-fed dams seen between the first and second week of pregnancy (Figure 3.1C).

The TD-NMR measurements are equally spaced apart by one week and are not matched to the gestational day of pregnancy. This means, depending on how quickly the mice plug the TD-NMR measurement may represent different time points in gestation which could vary by a maximum of four days. For example, post plug week two usually represents E12/13 but can also be any day between E10 and E14. There was, however, no significant difference between groups for the time taken to mate (Figure 3.1D) and there was no difference in the gestational day the final TD-NMR measurement was undertaken (Control, 13.1 ± 0.3; Obese, 12.6 ± 0.4; Ob-Ex, 12.3 ± 0.5; $p= 0.356$).

GWG between mating and E14 was recorded and showed an overall effect of maternal diet by one-way ANOVA (Figure 3.1E). E14 was the last day in pregnancy when bodyweights were consistently recorded in the dams from all three groups. Bodyweight was not recorded later in pregnancy so as to not disturb the mice close to delivery.

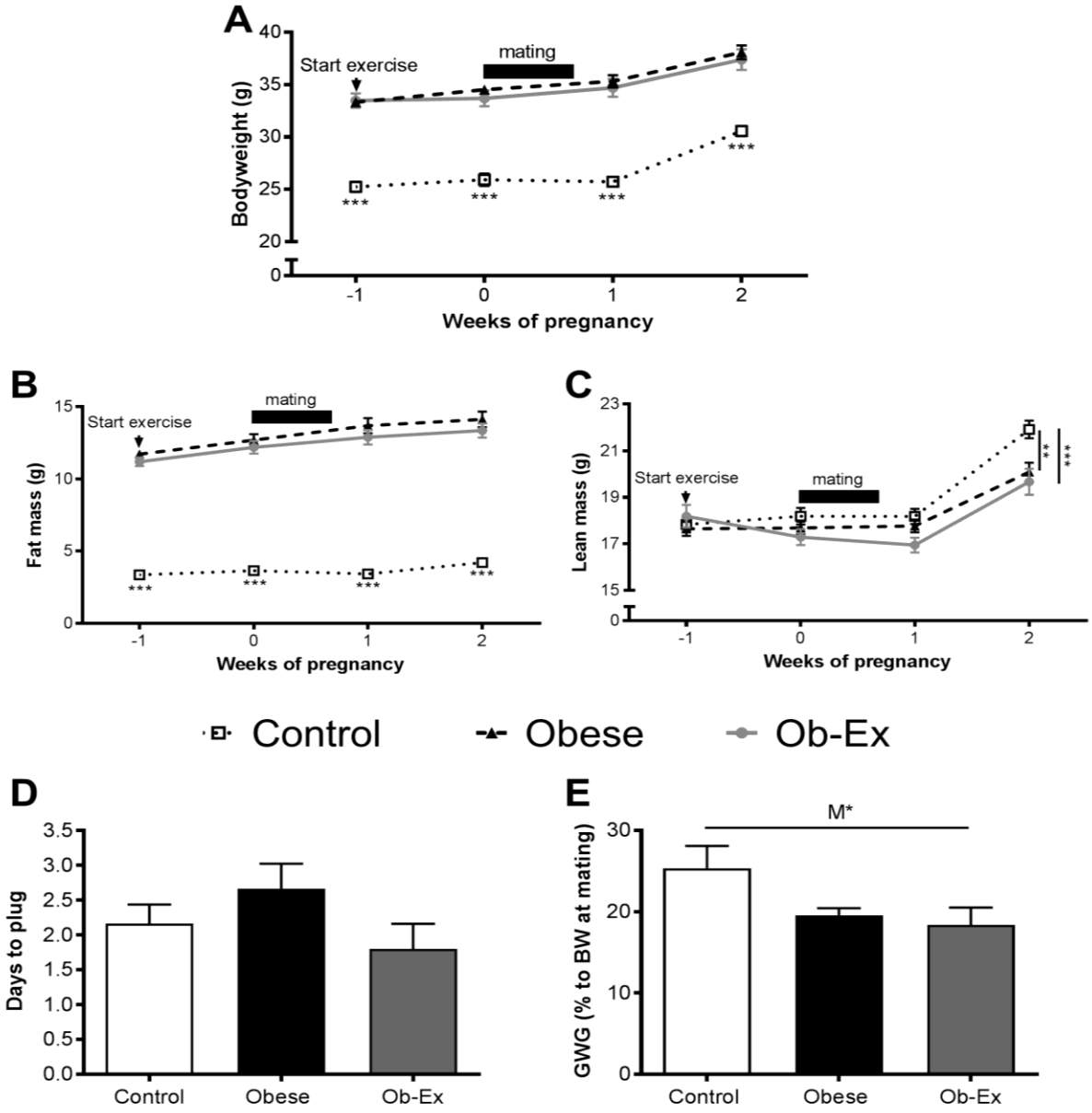


FIGURE 3.1: Maternal body composition from one week before and during pregnancy. TD-NMR measurements were started one week before mating when Obese and Ob-Ex dams reached the required 10 g fat. Ob-Ex dams also started the training week of exercise on this day. TD-NMR measurements continued weekly. A) Bodyweight, B) Fat mass and C) Lean mass. Control *n*= 12, Obese *n*= 12 and Ob-Ex *n*= 11. Two-way repeated measures ANOVA with Bonferroni post-hoc test; ** $p< 0.01$ and *** $p< 0.001$. D) Number of days until positive vaginal plug (maximum 5 days with stud male). Control *n*= 12, Obese *n*= 12 and Ob-Ex *n*= 10. E) GWG (E1-E14) relative to bodyweight at mating (E1). Control *n*= 11, Obese *n*= 12 and Ob-Ex *n*= 8. M*= overall effect of maternal lifestyle by one-way ANOVA ($p<0.05$).

Obesogenic diet-fed dams had a higher caloric intake during pregnancy then control diet-fed dams. There was no impact of maternal exercise intervention on daily caloric intake (Figure 3.2A). When the two components of the obesogenic diet (HFD pellet and condensed milk) were plotted separately, there was no difference in caloric intake during the intervention, between the non-exercised and exercise obese dams (Figure 3.2B). When caloric intake was plotted by the week of pregnancy there was no effect of maternal exercise or day of gestation on the intake of HFD pellet (Figure 3.2C). There was an effect of gestational day on the intake of condensed milk, showing that the consumption of the milk was reduced at the end of pregnancy. Overall there was no impact of maternal exercise on dam food intake during the intervention period.

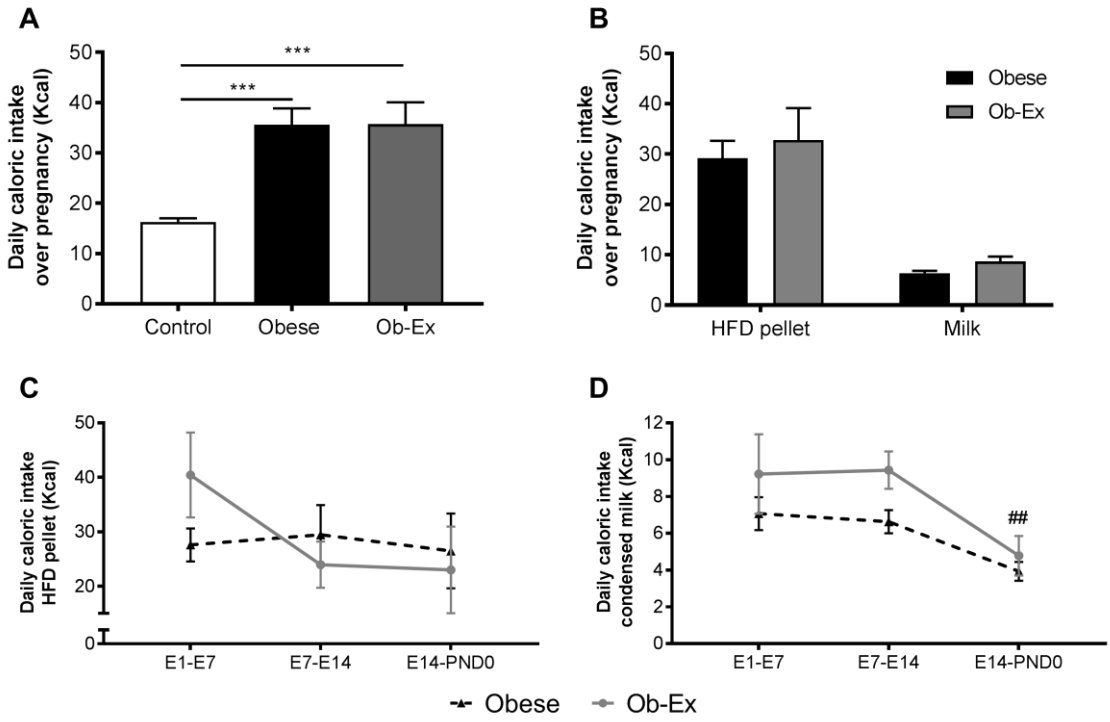


FIGURE 3.2: Maternal food intake during pregnancy. A) Average total daily caloric intake (from day of mating until day of birth) Control *n*= 12, Obese *n*= 10 and Ob-Ex *n*= 7. One-way ANOVA with Bonferroni post-hoc test; *** $p< 0.001$. B) Daily caloric intake of obesogenic diet-fed dams split into HFD pellet and condensed milk. C) Daily caloric intake of HFD pellet over pregnancy. E1 is the day of mating and PND0 is the day the pups were born. D) Daily caloric intake of condensed milk over pregnancy. Obese *n*= 10 and Ob-Ex *n*= 8. Two-way repeated measures ANOVA with Bonferroni post-hoc test; ## Significant effect $p< 0.01$ between E14-PND0 and other time points.

3.3.2 Litter and pup characteristics

Obese dams had a reduced number of pups in the litter and the maternal exercise did not prevent this (Figure 3.3A). There were no significant differences in the sex ratio of the litter between the groups (Figure 3.3B). The weight of PND2 pups was reduced after exposure to maternal obesity, the exercise intervention did not avert this reduction in pup weight (Figure 3.3C).

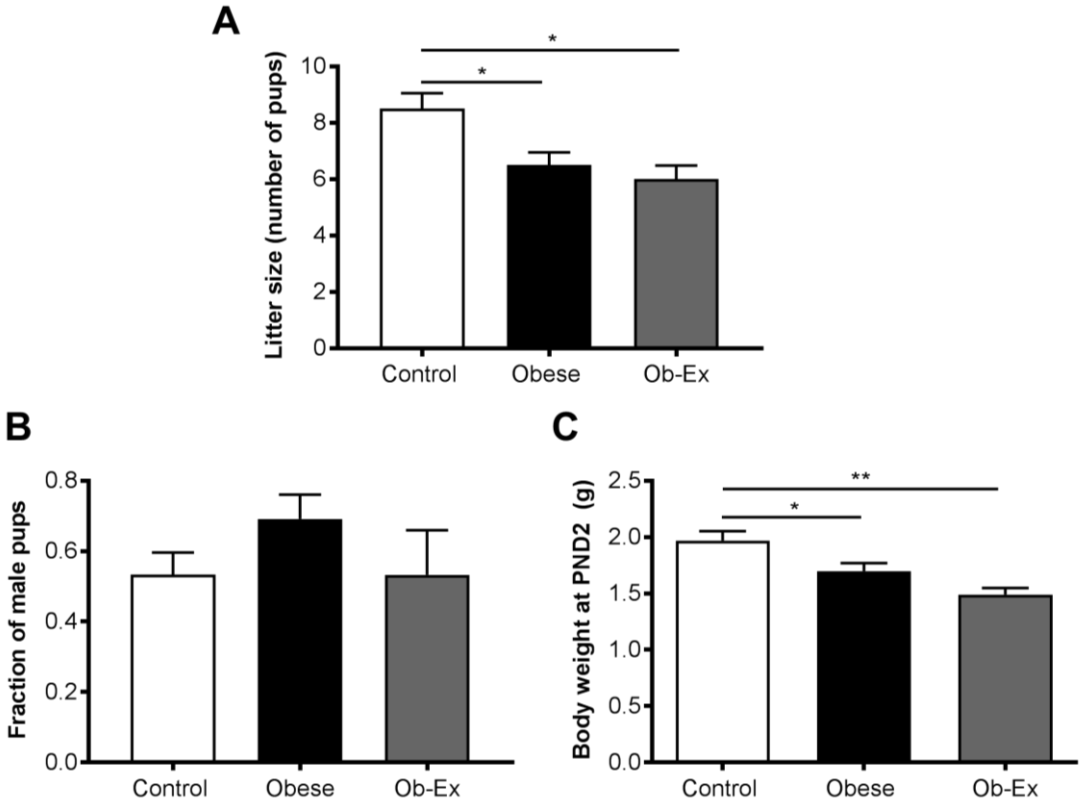


FIGURE 3.3: Litter size and weight. A) Total litter size, B) Fraction of male pups C) Average bodyweight of pup at PND2. Control *n*= 12, Obese *n*= 12 and Ob-Ex *n*= 7. One-way ANOVA with Bonferroni post-hoc test; * $p< 0.05$ and ** $p< 0.01$.

3.3.3 Lactation weights

Offspring from obese dams were heavier on PND14 and PND21 than the offspring from both control ($p< 0.001$) and obese-exercised dams ($p< 0.01$) (Figure 3.4A). At PND14, the bodyweight of offspring from obese-exercised dams was similar to that of offspring from control dams but there was a significant difference compared to offspring from obese dams ($p< 0.01$) (Figure 3.4A). By PND21 the bodyweight of the offspring in the intervention group tracks between the two other groups, there was still a significant difference compared to the offspring from obese dams ($p< 0.01$) but there was also increased bodyweight compared to offspring from control dams ($p< 0.01$) (Figure 3.4A). There was a significant interaction

between postnatal day and maternal lifestyle suggesting that age had the same effect on offspring of obese and obese-exercised dams, but that they were different to offspring of control dams. When FGR across lactation was calculated offspring from obese dams showed a significant increase. This was not prevented by maternal exercise intervention (Figure 3.4B).

Offspring of obese dams were heavier at three weeks of age compared to offspring from control dams; this was true for both male and female offspring. The offspring of obese-exercised dams did not have increased bodyweight compared to offspring of control dams. Their bodyweight was also not different, however, when compared to offspring of obese dams (Figure 3.4C+D).

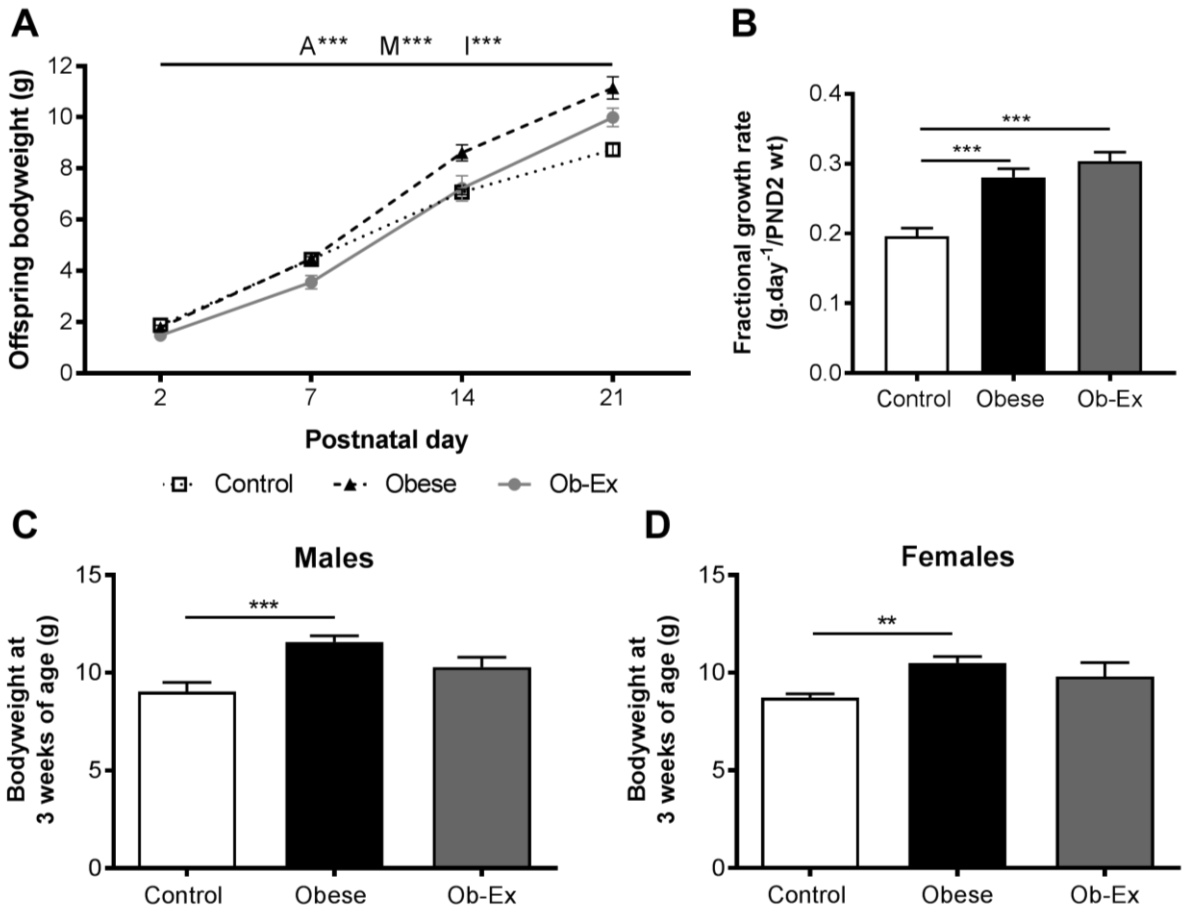


FIGURE 3.4: Lactation and weaning bodyweights. A) Offspring bodyweight on PND2, PND7, PND14 and PND21 (day of weaning). Control *n*= 9, Obese *n*= 9 and Ob-Ex *n*= 6. Two-way repeated measures ANOVA with Bonferroni post-hoc test; A*** = significant effect of age $p< 0.001$, M*** = significant effect of maternal lifestyle $p< 0.001$ and I*** = significant interaction $p< 0.001$. B) Fractional growth rate during lactation. Control *n*= 9, Obese *n*= 9 and Ob-Ex *n*= 6 C) Bodyweight at three weeks old of male (D) and female offspring. Control *n*= 9, Obese *n*= 9 and Ob-Ex *n*= 6. One-way ANOVA with Bonferroni post-hoc test; ** $p< 0.01$ and *** $p< 0.001$.

3.3.4 Maternal serological analysis at weaning

At weaning, maternal bodyweight was increased in the obesogenic diet-fed dams and there was no effect of the intervention (Figure 3.5A). Serum leptin was measured in 16 hour fasted serum and showed an increase (corresponding to the increased bodyweight) in the obese and obese-exercised dams. Serum leptin in the mother at weaning was not altered by the exercise intervention (Figure 3.5B).

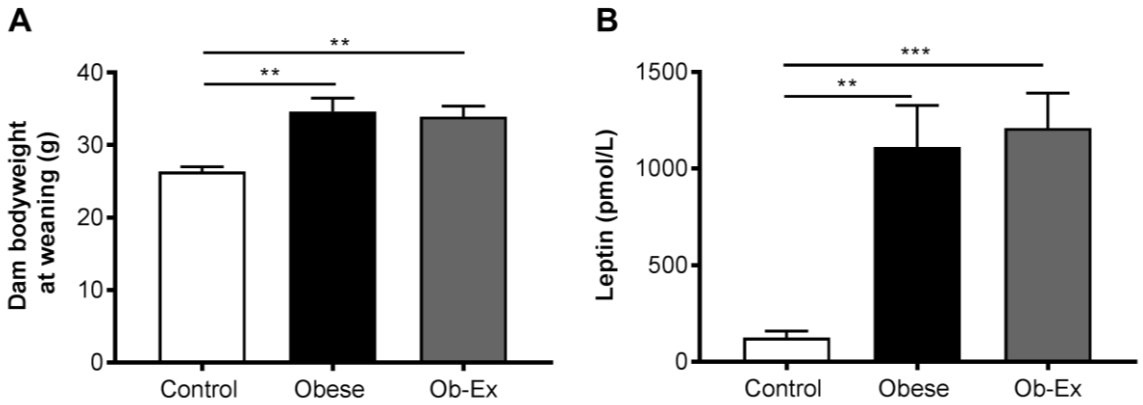
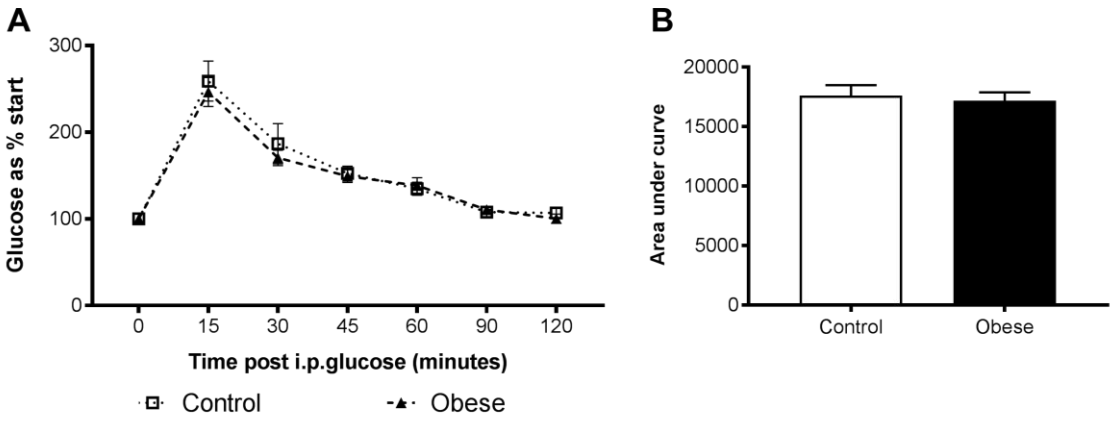


FIGURE 3.5: Dam bodyweight at weaning and serum leptin. A) Dam bodyweight at weaning. B) Dam leptin levels in 16 hour fasted serum. Control *n*= 7, Obese *n*= 4 and Ob-Ex *n*= 6. One-way ANOVA with Bonferroni post-hoc test; * $p < 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

It has already been determined that obese dams are hyperinsulinemic during pregnancy accompanied by impaired glucose tolerance (Fernandez-Twinn *et al.*, 2017). A GTT was performed on control and obese dams at weaning after a 16 hour overnight fast. There were no differences in glucose clearance after i.p. injection of 1 g/kg glucose (Figure 3.6A). This was reflected in the area under the curve analysis, as there was no difference between the groups (Figure 3.6B). Tail blood was collected at the 0 (baseline), 15 and 30 minutes. Plasma insulin was measured and was increased at all three time points. HOMA-IR was calculated after fasting, using the baseline sample (0 minute) taken before glucose injection and was increased in the obese dams (Figure 3.6C). This data was not available for exercised animals in this thesis. However, data provided by Dr Laura Kusinski and Dr Lisa Nicholas (Appendix AP2) shows that obese-exercised dams also do not have any further alteration in glucose tolerance at weaning.



C

	Plasma insulin (pmol/L)			*HOMA-IR*		
Time post i.p. glucose	*Control*	*Obese*	*P value*	*Control*	*Obese*	*P value*
0 minute	51.1 ± 2.9	112.8 ± 19.9	**0.048**	1.00 ± 0.05	2.24 ± 0.38	**0.040**
15 minutes	65.5 ± 5.1	179.1 ± 40.0	**0.031**	-	-	-
30 minutes	43.1 ± 3.8	110.9 ± 8.3	**<0.001**	-	-	-

FIGURE 3.6: Maternal GTT at weaning with matching plasma insulin. 0 minutes was at baseline following fasting and before i.p. injection. Control *n*= 5 and Obese *n*= 7. A) 16 hour fasted i.p. GTT. B) Area under curve analysis from GTT curve. C) Matching plasma insulin from the 0, 15 and 30 minute time points of the GTT and calculated HOMA-IR at fasting (0 minute). *P* values shown in table calculated unpaired t-test between Control and Obese group.

3.4 Discussion

The aim of this chapter was to determine the impact of exercise during an obese pregnancy on the mother and neonate up until weaning. The exercise intervention did not alter bodyweight or body composition throughout pregnancy. Reduced litter size and birthweight were not corrected and the accelerated growth profile in the lactation period was still present, but delayed, by the intervention. The offspring from the intervention group, however, did not show increased bodyweight at three weeks of age which was seen after exposure to maternal obesity. It has been shown previously that maternal glucose tolerance and hyperinsulinemia is present in obese dams during pregnancy (Fernandez-Twinn *et al.*, 2017). The data from this thesis has shown that this impaired glucose homeostasis was not maintained at weaning, and therefore the model has a gestational diabetic phenotype that is resolved when the dam is no longer pregnant.

3.4.1 Dam data

By mating, dams had been fed the obesogenic diet for around 15 weeks and this was sufficient to induce obesity. Obesogenic diet-fed dams therefore had increased bodyweight and fat mass throughout pregnancy and lactation. The increased bodyweight resulted from increased fat mass. The moderate intensity of the exercise intervention and the fact the animals were fed *ad libitum* meant that the dam's bodyweight and fat mass was not altered throughout the intervention. It is also important to note that during the week before mating the exercise protocol did not cause any significant weight loss, as periconceptional weight loss through maternal undernutrition has been shown to result in undesirable changes in fetal and neonatal measures (Matusiak *et al.*, 2014). Alongside no changes in body composition, there was also no benefit of maternal exercise on GWG (E1-E14) in obesogenic diet-fed dams. Overall, this model resulted in dams fed the obesogenic diet being heavier, with an increase in adiposity and is therefore a successful model of maternal obesity.

As the TD-NMR measurements were carried out weekly and were not matched to the day of gestation, the number of days to plug could have an impact on the last two TD-NMR measurements. The gestational day the TD-NMR measurement was carried out could vary by up to four days if the dam does not plug until the final day of mating (*Note*: Non-pregnant female mice were placed with stud males for a maximum of five days). To account for this possible confounding factor, the number of days to plug was plotted and there was no difference. This does not, therefore, account for the differences in the TD-NMR measurements between groups. During the second week of pregnancy, the lean mass of the obesogenic diet-fed dams was reduced and this was irrespective of if the dam was exercised. As TD-NMR measurements cannot distinguish between the body composition of the dam and her pups, this deficit could be accounted by the fact that both Obese and Ob-Ex groups had a reduced litter size at birth and a decreased PND2 pup weight.

The intervention was not continued into lactation; at the end of lactation, bodyweight was still increased in the obesogenic diet-fed dams with no effect of the prior exercise intervention. Maternal exercise did not alter serum leptin levels in pregnancy, which was measured shortly after the exercise intervention (at E19); where data showed an increase in obesogenic diet-fed dams and this was not ameliorated by the exercise intervention (Fernandez-Twinn *et al.*, 2017). Serum leptin was increased in samples from 16 hour fasted dams and this is consistent with a corresponding increase in fat mass (Shimizu *et al.*, 1997; Ruhl *et al.*, 2007). Body

composition of dams at the end of lactation was not determined in this study, but it has been shown in previous cohorts that obese dams had increased total body fat both in absolute terms and as a percentage of bodyweight at the end of lactation (Blackmore *et al.*, 2014; Fernandez-Twinn *et al.*, 2014). The lack of change in response to maternal exercise in these measured outcomes does not mean that the intervention had no impact on the dam. Previously published research from the lab has shown that maternal exercise prevented impaired glucose homeostasis and hyperinsulinemia during pregnancy (at E18) (Fernandez-Twinn *et al.*, 2017). It is important to identify maternal factors altered by obesity during pregnancy, as well as factors that have been corrected by the maternal exercise intervention as this will allow the identification of potential "programming factors" that could mediate offspring outcomes (shown in the subsequent chapters). In this case, offspring outcomes that are reversed by maternal exercise cannot be mediated by a reduction in the mother's adiposity but could instead be mediated by the improvement in glucose handling and insulin resistance during pregnancy.

A GDM-like phenotype

It has been shown by us (Fernandez-Twinn *et al.*, 2017) and in a separate study that uses the same mouse model (Samuelsson *et al.*, 2008), that there is impaired glucose tolerance and hyperinsulinemia in obese dams during pregnancy. This impairment in glucose tolerance appears to be confined to pregnancy, as by the end of lactation there was no longer any difference in glucose tolerance in the obese dams compared to controls. This suggests a GDM-like phenotype where obesity in the dam is not enough to impair glucose tolerance alone and differences are only seen when paired with the demands of pregnancy. During pregnancy, adjustments in glucose homeostasis alongside other significant physiological and metabolic changes ensure the increasing demand of supplying nutrients to placenta and developing fetus are met (Di Cianni *et al.*, 2003).

Hyperinsulinemia is maintained at weaning in the obese dams (this was measured in 16 hour fasted plasma). Plasma samples collected during the glucose peak of the GTT (15 and 30 minutes post i.p. glucose) also had elevated insulin levels in the obese dams. This could indicate that the obese dams require higher insulin levels to handle the glucose bolus given to maintain glycemia in the non-pregnant state. HOMA-IR index could be calculated as matching plasma glucose and insulin was available and this showed increased HOMA-IR following fasting, indicating increased insulin resistance. Insulin resistance is thought to

represent a starting point in the pathogenic progression of diabetes (Groop, 2000). Impaired glucose tolerance and diabetes won't be present if the β-cells can produce enough insulin to maintain blood glucose within normal range. When this is no longer possible there will be disease progression. Therefore during pregnancy when further insulin resistance occurs, the obese dams had impaired glucose clearance possibly because their β-cells are now struggling to produce enough insulin to maintain glucose homeostasis.

3.4.2 Neonatal offspring growth

Pups from obese dams had decreased bodyweight at PND2 and were also born in smaller litters. Low birthweight in offspring of obese dams has previously been shown in both rats (Howie *et al.*, 2009) and mice (Zheng *et al.*, 2014). In human obese pregnancies women are just as likely to have offspring born SGA, as they are to have LGA babies (Rajasingam *et al.*, 2009). Maternal exercise of the obese dams did not cause any additional change in neonate weight or litter size. An epidemiology study of exercise during a lean pregnancy resulted in lower birthweight and BMI (Hopkins *et al.*, 2010). In our obese dams, exercise did not appear to further reduce PND2 offspring weight as there was no difference between offspring of obese and obese-exercised dams; this suggests a difference in how fetal growth profiles are affected by exercise between a lean and obese pregnancy.

Despite offspring from obese dams being smaller at PND2, they became heavier (vs. control) from PND14 onwards and had increased FGR during lactation, suggesting an alteration in their growth profile and possible postnatal 'catch up growth'. Body composition analysis by TD-NMR is not possible during this period due to their small size, therefore it is not known if this increased weight in the postnatal period is due to changes in lean mass or fat mass. This resulted in an increased bodyweight at three weeks of age in male and female offspring from this group. The offspring of obese-exercised dams were also smaller at PND2 but did not show increased bodyweight during lactation until PND21 suggesting the accelerated growth occurs later in lactation. FGR was also increased in these offspring, again suggesting an accelerated growth rate; however when weights were plotted individually at three weeks of age, male and female offspring of obese-exercised dams had bodyweights that were similar to control. Alterations in the growth profile during lactation can be harmful, with studies showing that 'catch up growth' is associated with CVD and insulin resistance in adult life (Cianfarani, Germani and Branca, 1999; Eriksson *et al.*, 1999).

3.4.3 Translatability of the intervention

Adherence, in humans, to an intervention that involves a change in behaviour is a major limitation with the lowest adherence reported to be 16% (Russo *et al.*, 2015). This still presents a challenge in implementing a successful intervention. Interventions during pregnancy can be complicated by the reported side effects of pregnancy such as nausea and fatigue. The exercise protocol was designed to be of moderate intensity for only 20 minutes a day, five days a week and should, therefore, be a realistic goal for obese pregnant women.

Primary outcomes in the mother are not always met with exercise and dietary-based interventions, however differences in other outcomes are recorded and the potential benefit of the interventions on the infant and long-term health of the mother has yet to be assessed in most current human studies. For example, the UPBEAT trial demonstrated reduced infant adiposity at six months of age in the intervention group and a sustained improvement in the mother's diet at six months postpartum (Patel, Godfrey *et al.*, 2017). Increasing the intensity and duration of the intervention to maximize the impact is probably not going to be realistic in an obese population. Therefore, having an intervention that can have a positive impact without a change in maternal body composition or GWG is important. The impact of this intervention on offspring outcomes and its success will be assessed in the subsequent chapters.

3.4.4 Conclusions

Dams fed an obesogenic diet prior to conception can be considered obese at mating and throughout pregnancy and lactation; this increase in bodyweight was attributed to an increase in fat mass. Maternal exercise intervention did not alter bodyweight or body composition throughout pregnancy when compared to non-exercised obese dams. At the end of lactation, obesogenic diet-fed dams remained heavier and had increased serum leptin. Obese dams had normal glucose tolerance but showed hyperinsulinemia and insulin resistance at weaning. Alongside the previous published knowledge that there is impaired glucose tolerance during late pregnancy, this suggests the phenotype is similar to GDM. Pups born to obese dams were smaller at PND2 but then had accelerated postnatal growth and were heavier by weaning. The exercise intervention did not prevent pups being smaller at PND2 and these pups continued to have accelerated growth during lactation period.

3.4.5 Summary of key findings

Results in this chapter have shown that:

- Feeding of the obesogenic diet to the dams doubled the daily caloric intake and dams were fed respective diets for approximately 15 weeks before the start of the experimental protocol.

- One week before pregnancy, when the Ob-Ex group started the exercise-training week, the obesogenic diet-fed dams were roughly 30% heavier and had over double the adiposity. The exercise intervention did not alter body composition of the dams.

- At weaning, maternal obesity caused fasting hyperinsulinemia and insulin resistance as indicate by the increased HOMA-IR.

- Maternal obesity decreased the pup weight on PND2 and this was not prevented by the exercise intervention.

- Pups of obese dams, irrespective of if the obese dam was exercised, had increased FGR in the lactational period and were heavier at weaning, possibly indicating a ‘catch up growth’ phenotype.

4. Cardiovascular phenotype in young adult male offspring

4.1 Introduction

4.1.1 Importance of CVD prevention

The incidence of mortality from CVD fell by 68% between 1980 and 2013 (Bhatnagar *et al.*, 2016) however there are more people living with the effects and impact of CVD than ever before (Koch *et al.*, 2015). This has been attributed to massive improvements in both prevention and treatment of cardiovascular events; for example improved control and management of hypertension through the use of beta blockers, the reduction in circulating cholesterol levels through administration of statins and the development and rapid use of thrombolysis or stents in an ischaemic event to limit tissue death (Mensah *et al.*, 2017). With an ageing population, the incidence of CVD is expected to increase as people above 65 years of age have the highest prevalence for all CVD. This will have large implications for the healthcare services; a study predicting medical costs for CVD in the US suggest it will rise by 200% by 2030 (Heidenreich *et al.*, 2011).

Prevention strategies have focused specifically on attenuating disease risk by reducing an individual's modifiable risk factors, for example through an improvement in current lifestyle choices (U.S. Department of Health and Human Services, 2010; Holmes *et al.*, 2014). However, this strategy does not target prevention of the risk of cardiometabolic disease that is established during fetal development through exposure to an adverse *in utero* environment such as maternal obesity. Maternal obesity impacts the offspring's cardiovascular health through programmed mechanisms, this is described in human and animal studies in Section 1.8-1.9. Instead current strategies target a window (adulthood) where disease pathologies may already be developed and does not take into account the disease risk already attributed to individuals at birth. Failing to address this particular risk could greatly increase the burden for CVD in the next generation. A prevention strategy is needed to target the *in utero* cardiovascular health of the unborn offspring.

4.1.2 Left ventricular cardiac hypertrophy

LV hypertrophy occurs to normalise wall tension in the heart in response to pressure/volume overload. It is not a pre-requisite that this will result in cardiac dysfunction and is a common compensatory response to the physiological stressors of pregnancy and exercise (Nakamura and Sadoshima, 2018). This is known as physiological cardiac hypertrophy and it is characterised by increases in heart weight, cardiomyocyte cell size, protein synthesis and cardiac output. It is often reversible when the initial stressor is removed (Agrawal *et al.*, 2010). However cardiac hypertrophy can also be pathological in nature and this is accompanied by adverse cardiovascular events such as arrhythmias, heart failure and death (Nakamura and Sadoshima, 2018). Risk factors for the development of LV hypertrophy include age, obesity, diabetes, hypercholesterolemia and prior myocardial infarction (Artham *et al.*, 2009). Factors of the neurohumoral system have also been implicated including the renin-angiotensin and adrenergic systems, insulin, catecholamines and other growth factors (Heineke and Molkentin, 2006).

The Framingham study recruited residents aged 30-60 years old, from the town of Framingham, Massachusetts, USA for a landmark long-term study that aimed to identify common risk factors for the development of CVD. The study used echocardiographic determination of LV mass to offer prognostic implications (Levy *et al.*, 1990). LV hypertrophy has been shown to hinder function through a reduction in stroke volume and rapid ventricular filling volume and time (Folkow, 1978; Smith *et al.*, 1985).

Increased LV mass and hypertrophy have been shown, in the Cardiovascular Health Study, to be a risk factor for depressed LV ejection fraction (Drazner *et al.*, 2005). The mechanism by which this happens is not well understood (Artham *et al.*, 2009). LV hypertrophy is also associated with interstitial fibrosis; another myocardial structural change that is known to impact function (Weber and Brilla, 1993). Specifically, hypertrophied tissue contains increased amounts of collagen and interstitial fibrosis and this has been shown to impact diastolic function (Artham *et al.*, 2009). Other complications of LV hypertrophy are ventricular arrhythmias (McLenachan *et al.*, 1989) and atrial fibrillation (Verdecchia *et al.*, 2003), both of which have been linked to sudden cardiac death. In another study, hypertrophied cells had altered ventricular physiology through a decrease in outward current caused by delayed rectifier channels (Kleiman and Houser, 1990).

LV hypertrophy and hypertension

Although LV hypertrophy does occur in other rheumatic and coronary heart disease, hypertension is the chief precursor for the condition with elevated SBP, as opposed to diastolic, having the bigger impact. LV hypertrophy is a common consequence of hypertension as it acts as a fundamental adaptation to the pressure overload mechanism. This common adaptation allows the heart to maintain cardiac output during increases in afterload caused by increased arterial pressure (Dahlof, 2001). This may produce a stable period of compensation but eventually decompensation will occur leaving the ventricle unable to cope and leads to heart failure. The risk of death or fatal complications is increased 2-4 fold with LV hypertrophy (independent of age, sex and other risk factors) (Kannel, Dannenberg and Levy, 1987; Levy *et al.*, 1990).

Data from the Framingham study showed that 30% of those with echocardiography-detected LV hypertrophy would go on to have a cardiovascular event within five years. LV hypertrophy is a strong independent predictor of future cardiovascular events (Dunn *et al.*, 1990). Moreover, LV hypertrophy also increases the risk of coronary heart disease, myocardial infarction, stroke and heart failure independently of BP; the contribution of this risk is calculated to exceed the risk of the accompanying hypertension (Kannel, Dannenberg and Levy, 1987). These findings were supported in a study undertaken in a population of patients with hypertension that were more diverse racially and in socioeconomic background than the Framingham study. This study demonstrated that the increment in LV mass became an independent predictor of cardiovascular death including myocardial infarction and heart failure (Koren *et al.*, 1991).

Population based studies confirm that hypertension increases an individual's risk of further cardiovascular complications by 2-3 times (Padwal, Straus and McAlister, 2001). Hypertension has been implicated in 39% of cases of heart failure in men and 59% in women suggesting its involvement in the aetiology of progressive cardiovascular disease (Levy *et al.*, 1996).

Reversal of LV hypertrophy

The use of anti-hypertensive therapy can regress LV hypertrophy and has been shown to reduce CVD risk, independent of BP lowering (Nagano *et al.*, 1991). Contractility has also been shown to be improved even after anti-hypertensive therapies were stopped (Schmieder *et al.*, 1989). Further studies demonstrated that blunting of the hypertrophic response is

associated with preserved cardiovascular function (Schmieder *et al.*, 1989) and improved survival with a reduced morbidity and mortality (Prineas *et al.*, 2001). The greater the reduction in LV hypertrophy the greater the reduction in major cardiovascular events (Okin *et al.*, 2004). Since there is little understanding about the mechanism linking LV hypertrophy and poor cardiac function, the reason for a reduction in LV hypertrophy to improve clinical outcomes is similarly unknown.

4.1.3 Pathological cardiac hypertrophy markers

It is well described that during cardiac stress such as hemodynamic overload (Distefano and Sciacca, 2012) there is a re-expression of fetal genes. This alteration in gene expression is initially compensatory to maintain cardiac output, but decompensation can occur with persistent expression, and this may lead to heart failure (Distefano and Sciacca, 2012).

Natriuretic peptides

The *Nppa* gene encodes atrial natriuretic peptide (ANP) and *Nppb* encodes brain natriuretic peptide (BNP); both are circulating hormones that play a role in the regulation of intravascular blood volume and vascular tone. *Nppa* preferentially binds to the guanylyl cyclase-linked natriuretic peptide receptor-A (NPR-A) but can also bind to natriuretic peptide receptor-C which is not guanylyl linked and instead acts to clear the peptides from the circulation (Nishikimi, Maeda and Matsuoka, 2006). During embryonic development *Nppa* is expressed in both atria and ventricles, however after birth, expression is mainly confined to the atria within the mature myocardium (Chien *et al.*, 2016). After induction of ventricular hypertrophy, *Nppa* is re-expressed in ventricular tissue. The induction of *Nppa* is a highly conserved feature of ventricular hypertrophy and this has been demonstrated in many animal models in response to a variety of stimuli such as hormone, pressure and volume overload and in genetic models (Chien *et al.*, 2016). Mice lacking the NPR-A demonstrate an elevated ventricular mass and hypertension indicating this peptide may have a role in inhibiting cardiac hypertrophy (Oliver *et al.*, 1997). This was then investigated *in vitro,* rat neonatal ventricular myocytes treated with endogenous ANP showed inhibition of hypertrophy (Horio *et al.*, 2000). These peptides cause effects that extend beyond the heart for example diuresis (increased urine production), natriuresis (increased sodium excretion), vasodilation and inhibition of the renin- secretion and aldosterone system (Nishikimi, Maeda and Matsuoka, 2006).

Myosin heavy chain switch

α and β myosin heavy chain (MYC) are two functionally distinct cardiac MYC isoforms; α-MYC (*Myh6*) is the adult form and β-MYC (*Myh7*) the predominant fetal isoform (Holubarsch *et al.*, 1985). Up-regulation of β-MYC is an early and sensitive marker of cardiac hypertrophy (Pandya and Smithies, 2011).

β-MHC is characterised by lower adenosine triphosphatase activity and lower filament sliding velocity, but can generate cross-bridge force with a higher economy of energy consumption than α-MHC (Holubarsch *et al.*, 1985). This suggests that a shift from α- to β-MHC might be an adaptive response to cardiac stress in order to preserve energy. It is therefore conceivable that the decrease in contractile function due to increased β-MHC might outweigh the benefits of improved economy and ultimately dictate clinical outcome (Krenz and Robbins, 2004). This shift in MYC ratio is common in hypothyroidism, triiodothyronine is able to bind to the thyroid hormone responsive element (TRE) of the *Myh6* gene causing its upregulation. It can also bind to the TRE located in the *Myh7* gene but this causes repression (Kuwahara, Nishikimi and Nakao, 2012). The subsequent increased β-MHC:α-MHC ratio resulted in impaired systolic cardiac dysfunction measured by magnetic resonance imaging (Chen *et al.*, 2001).

4.1.4 Cardiac inotropic response to changes in workload

Modulation of calcium pathways is critical for the heart to adapt to changing workloads (Chung *et al.*, 2016; Eisner *et al.*, 2017). Calcium (Ca^{2+}) is at the centre of the excitation-contraction coupling in the heart. The regulation of contraction of individual cardiomyocytes (the coordination of which is critical for effective pumping) is controlled by Ca^{2+} entry and release from a specialist organelle called the sarcoplasmic reticulum (SR). Ca^{2+} is stored in the SR of cardiomyocytes. The contraction cycle is triggered by the depolarisation of the sarcolemma, which activates the voltage gated L-type Ca^{2+} channels, and the subsequent Ca^{2+} entry via these channels into the cardiomyocyte starts the release of Ca^{2+} from the SR via ryanodine receptors (Woodcock and Matkovich, 2005).

The increase in intracellular Ca^{2+} then binds to Troponin C of the myofilament machinery and relieves the inhibition of the myosin and actin interaction. Tropomyosin is a specialised rod shaped protein that binds to the length of the actin filament to stabilise and stiffen it. Troponin T positions the myofilament machinery complex on the thin actin filament by binding to Tropomyosin. Troponin I is a polypeptide in the complex involved in Ca^{2+} regulation, it has

an inhibitory role while it is bound to actin. The increase in intracellular Ca^{2+} after Ca^{2+} entry allows Ca^{2+} to bind to Troponin C which releases the inhibitory interaction of Troponin I. This causes a shift in Tropomyosin to allow the binding of the myosin head to the actin filament which results in contraction (Bers, 2002). Ca^{2+} return to the SR is achieved in two ways, via the sarcoplasmic reticulum Ca^{2+} transporter (SERCA) (Bers, Eisner and Valdivia, 2003) and through Na^{+}/Ca^{2+} exchanger (Hilgemann, 2004).

The force and/or rate of contraction has to be modifiable to allow the heart to increase the cardiac output in response to changes to workload. This is achieved by enhancing the Ca^{2+} mediated mechanisms described above and occurs through the activation of sympathetic nervous system. Phosphorylation of Troponin I, through sympathetic activation, decreases the Ca^{2+} sensitivity of Troponin C which causes an increased off-rate of Ca^{2+}, to allow accelerated cardiac relaxation (Bodor *et al.*, 1997; Zakhary *et al.*, 1999). Phosphorylation occurs through increased β-adrenergic receptor activity leading to activation of protein kinase A/C.

4.1.5 Exercise as an intervention

Exercise is an effective intervention at improving one's own metabolic health and therefore presents a promising candidate for intervening during an obese pregnancy, as it is known that improving the mother's metabolism will also impact the future offspring. As shown in Section 1.10 many human intervention studies have used exercise in an attempt to improve mother's health as well as fetal and neonatal outcomes. Most of these studies report only on immediate effects during pregnancy and at the point of delivery. Recent findings from the UPBEAT study have shown that increased physical activity and a reduction in glycemic load during pregnancy reduced offspring adiposity at six months of age (Patel, Godfrey *et al.*, 2017). However, due to the long-term nature of CVD and the relative infancy of such intervention studies, data on such outcomes in humans will not be known for many years. There is therefore a need to model such intervention strategies in animals to longitudinally determine the cardiometabolic effects on the offspring.

4.1.6 Aim of the chapter

The aim of this chapter was therefore to use a mouse model of maternal diet-induced obesity to test the effectiveness of an exercise intervention (treadmill running) during obese pregnancy at preventing programmed cardiovascular dysfunction in the adult male offspring and identifying underlying mechanisms.

4.2 Methods

4.2.1 Animals

Full details of the animal model with experimental schematic is in Sections 2.1 and 2.2. Weekly TD-NMR measurements were carried out on the male offspring from four weeks until eight weeks of age. At eight weeks of age tail blood glucose was measured after a 16 hour fast (AlphaTRAK 2, Zoetis, USA). Mice were then killed by rising CO_2 concentration. At post mortem, blood was collected by cardiac puncture before tissue was collected and weighed before being snap frozen on dry ice. All samples were stored at -80°C until analysis took place. Serum analysis is described in Section 2.3.

4.2.2 Cardiomyocyte area analysis: WGA and CellD

Immersion-fixed hearts were processed and sectioned at 10 µm. Using a previously published method (Blackmore *et al.*, 2014), three mid-cardiac sections from each heart were selected for staining; six hearts (from six independent litters) were analysed per experimental group. The experimental group was blinded to the analyser. Wheat germ agglutinin [TexasRed-X conjugate, (Molecular Probes, Life Technologies, USA)] was used to stain cardiomyocyte cell borders. Sections were de-waxed using xylene and re-hydrated by immersing in decreasing strengths of ethanol. Sections were washed with phosphate buffered saline (PBS) before incubating with 10 µg/ml of agglutinin (in PBS) while gently rocking in darkness for 2 hours at room temperature. Slides were washed with PBS and air-dried before the addition of Vectashield mounting medium with 4'-6-Diamidino-2-phenylindole (Vector Laboratories, UK). Five images moving down the left ventricle were randomly taken using a Leica SP8 confocal microscope (Leica, Germany) with the 40x oil immersion objective. Images were exported with a scale bar from the confocal microscope in a TIFF format. Images were analysed in CellD software (Olympus, Japan). After setting the scale, the polygon tool was used to manually circumscribe around the cell borders for all cells that were within cross section, complete and visible within image. Analysis was carried out on at least three images per heart section, and three heart sections were analysed per animal.

4.2.3 PCR: primer sequences

The PCR protocol including how primers were designed is described in Section 2.6. The primer sequences used in this chapter are shown in Table 4.1.

TABLE 4.1: Primer sequences for PCR. Primers in **bold** were used as housekeeper genes for normalisation

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Gapdh	AGAGTGTTTCCTCGTCCCGT	GCTGGGGAAGTAACTGGAG
Nppa	ATCGGAGCCTACGAAGATCCA	TTCGGTACCGGAAGCTGTT
Nppb	AGTCCTTCGGTCTCAAGGCA	AACTTCAGTGCGTTACAGCC
Myh6	CTGTTCCTCTCTCCGTCCAG	ATTCTGTCACTCAAACTCTGGTTA
Myh7	GCCAACTATGCTGGAGCTGA	GCAGACACGGTCTGAAAGGA
Ehd2	GGTGCAAAAGAGAGAGTGAGC	AGAGCAAGATGATGAGGTCCA
Cat	TCACTCAGGTGCGGACATTC	TAGTCAGGGTGGACGTCAGGT
Sod2	GGAGCAAGGTCGCTTACAGA	GCGGAATAAGGCCTGTTGTT
Gpx3	TTCTGAAGAACTCCTGCCCT	CCAGAGGATGTAATGGATCT
Nrf2	AGGTTGCCCACATTCCCAAA	AGGGCAAGCGACTCATGG

4.2.4 PicroSirius red stain: Fibrosis analysis

Paraffin embedded mid-cardiac sections cut at 10 μm were de-waxed using the protocol previously mentioned (Section 4.2.2). Slides were submerged in PicroSirius red stain (Pioneer Research Chemicals Ltd, UK) for one hour with agitation. Slides were then plunged very briefly in and out of water and then allowed to dry face down over a downdraught bench to prevent pooling of stain at thicker edges of sections. Slides were placed in xylene for two minutes, this step was repeated before mounting with Pertex® mounting medium. After drying with mountant overnight slides were imaged using AxioscanZ1 slide scanner (Zeiss, Germany). The experimental group was blinded to the analyser. A 12 by 12 grid was superimposed onto the image. 10 grids per mid-cardiac section were selected randomly for analysis in ImageJ. RGB stack function was performed and an appropriate threshold was set to comprehensively allow the software to detect the positive stain. This threshold was then used in all further analysis. Area fraction of positive stain was then quantified for each of the 10 randomly chosen grids. This was taken to represent a measure of % fibrosis in the mid-cardiac section.

4.2.5 Non-invasive blood pressure measurement

Full detailed methodology can be found in Section 2.4.

4.2.6 Echocardiography: Aortic width analysis

Full methodology is described in Section 2.5. Ascending aortic diameter was measured from captured EKV™ files and the width from wall to wall at mid-systole and diastole was measured within 1 mm of the aortic valve (Figure 4.1).

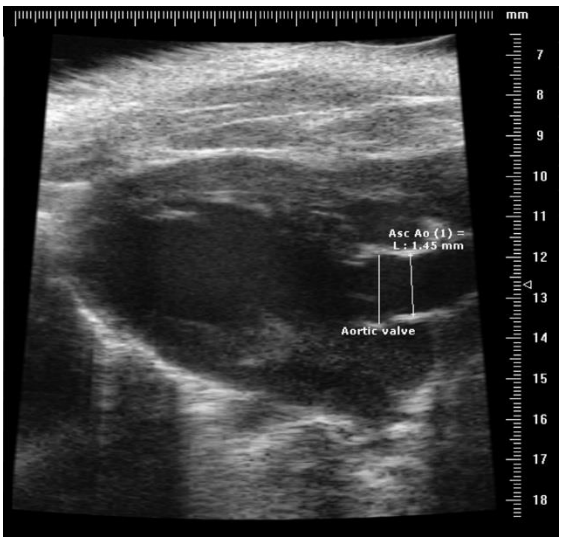


FIGURE 4.1: EKV™ image of the LV showing measurements of ascending aorta width. Ascending aorta (Asc Ao) width was measured within 1 mm of the aortic valve (labelled on figure) at both end-systole (pictured) and end-diastole (not shown).

4.2.7 Western Blotting

Methodology for western blotting is described in Section 2.8. Coomassie stained gel shows equal protein loading (Figure 4.2). All primary antibodies were used at a 1:1,000 dilution and were obtained from Cell Signaling Technology (MA, USA): Tropomyosin (#3990S rabbit monoclonal), Troponin I (#4002S rabbit polyclonal), phosphorylated (Ser23/24) Troponin I (phos-Troponin I) (#4004S rabbit polyclonal) and ATP2A2/SERCA2 (#4388S rabbit polyclonal).

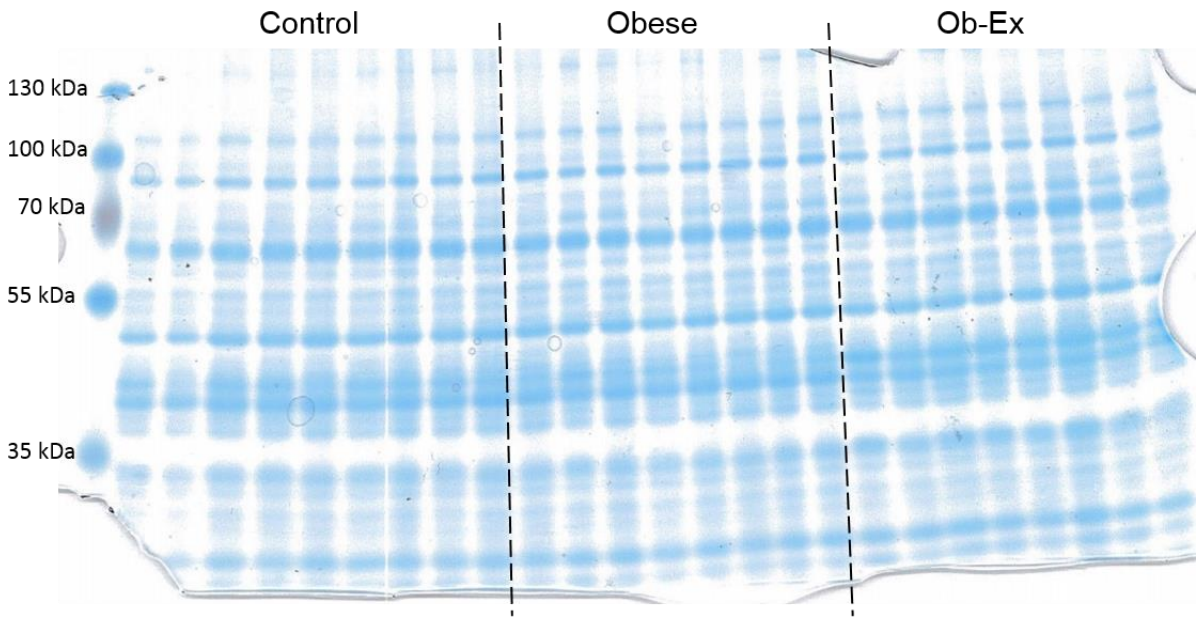


FIGURE 4.2: Coomassie stain of loaded gel to ensure protein loading. Control *n*= 9, Obese *n*= 8 and Ob-Ex *n*= 8.

4.2.8 Statistical analysis

TD-NMR measurements were analysed by two-way ANOVA with Bonferroni post-hoc test. When three groups were compared, one-way ANOVA with Bonferroni post-hoc test was used. Cell area data were analysed using a hierarchical linear model with random effects for individual animal, and an interactive effect between individual and section of origin (R statistical software package version 2.14.1 (R Foundation for Statistical Computing, Vienna, Austria). The model included maternal diet and maternal exercise as fixed effects. This structure accounted for the fact that multiple cell area measurements were obtained from multiple sections of the heart of each individual animal within the groups, and these data cannot be treated as fully independent. All data is presented as mean ± SEM.

4.3 Results

4.3.1 Body composition and organ weights

Bodyweight and body composition were assessed longitudinally (weekly). TD-NMR (to measure body composition) was carried out weekly from four weeks of age up until eight weeks of age. Due to missing data a correction for repeated measures could not be carried out therefore a two-way ANOVA followed by Bonferroni post-hoc when necessary was used to assess statistical significance. Age had a significant effect on bodyweight, fat mass and lean

mass (Figure 4.3A-C). Maternal lifestyle (accounting for maternal diet and if the dam was exercised) only had a significant impact on fat mass. The offspring of obese dams had more fat mass compared to control, with the biggest differences observed at seven and eight weeks of age (Figure 4.3B).

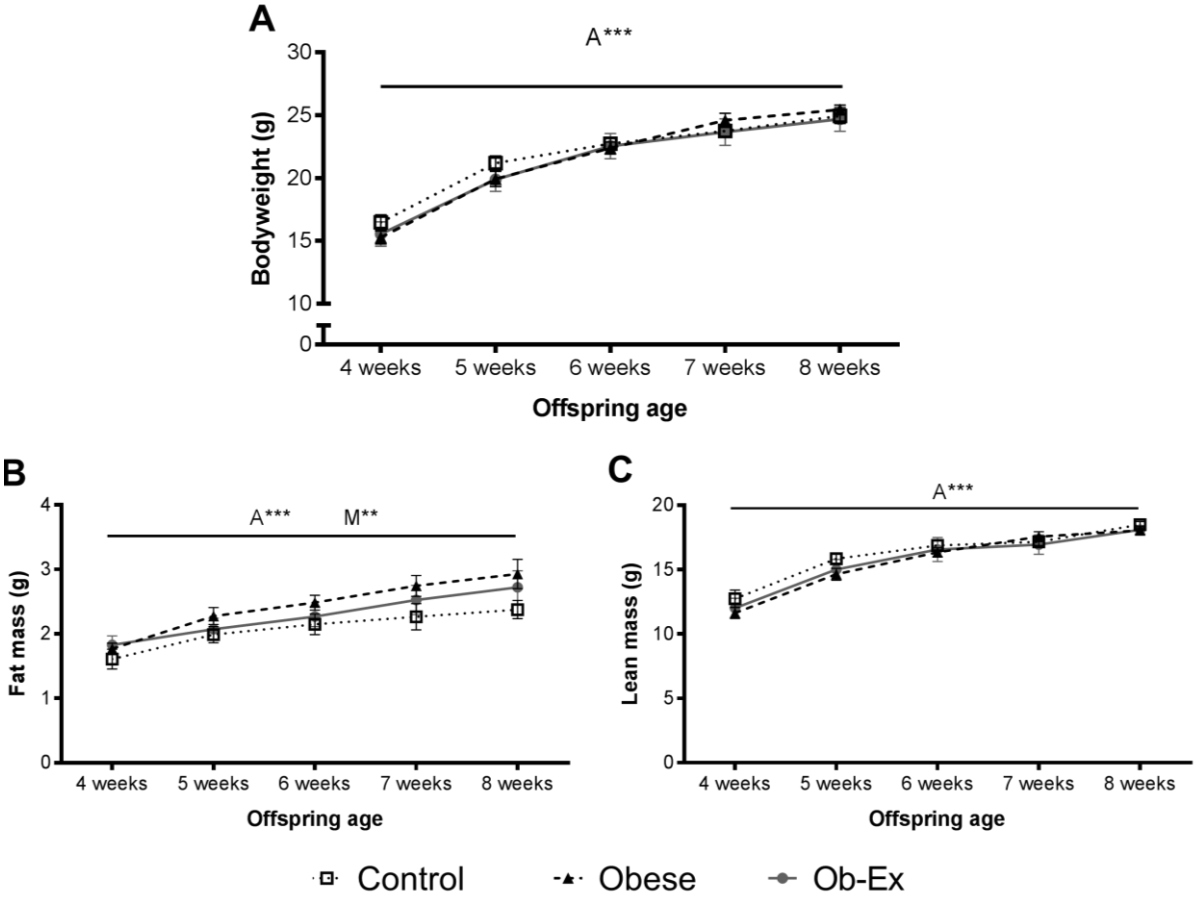


FIGURE 4.3: Longitudinal assessment of body composition. Body composition measured by TD-NMR from four weeks of age to eight weeks of age. TD-NMR measurements were carried out at the same time of the day each week (Morning) to account for different feeding behaviours in light/dark cycle. A) Bodyweight, B) Fat mass and C) Lean mass. Control n= 9, Obese n= 10 and Ob-Ex n= 6. Two-way ANOVA; A*** effect of age, p< 0.001 and M** effect of maternal lifestyle, p< 0.01.

When body composition was assessed at eight weeks of age there was no difference in bodyweight, fat mass and lean mass between groups (Table 4.2). Male offspring were fasted for 16 hours (overnight) prior to *post mortem* at eight weeks of age. There were no differences in the fasted bodyweight or in the bodyweight lost during the fast (Table 4.2). Despite the effect of maternal lifestyle in TDNMR fat mass there were no difference in the weights of the

fat depot (epididymal and retroperitoneal) (Table 4.2). There were no differences in other measured organ weights (Table 4.2).

TABLE 4.2: Body composition and organ weights in eight week old male offspring. TD-NMR measured body composition in the fed state. Data is presented mean ± SEM. Males were fasted for 16 hour before post mortem where organs were dissected and the wet tissue weight was recorded. *P* values calculated from one-way ANOVA.

Body composition + organ weights (g)	*Control*	*n*	*Obese*	*n*	*Ob-Ex*	*n*	*p value*
Fed bodyweight	25.5 ± 0.3	6	25.6 ± 0.3	6	25.6 ± 0.5	5	0.98
Fat mass	2.3 ± 0.1	6	3.1 ± 0.3	6	2.6 ± 0.3	5	0.06
Lean mass	18.5 ± 0.3	6	18.1 ± 0.3	6	18.1 ± 0.3	5	0.56
Fasted bodyweight	*22.8* ± 0.4	6	22.9 ± 0.4	8	22.7 ± 0.5	6	0.934
Bodyweight lost with fast	1.92 ± 0.19	6	2.24 ± 0.14	8	2.07 ± 0.12	6	0.696
Epididymal fat	0.24 ± 0.02	8	0.30 ± 0.03	9	0.28 ± 0.02	5	0.192
Retroperitoneal fat	0.05 ± 0.005	8	0.05 ± 0.003	9	0.06 ± 0.007	5	0.813
Liver	0.97 ± 0.04	8	0.98 ± 0.04	9	0.97 ± 0.03	5	0.954
Kidney; Left	0.17 ± 0.004	8	0.17 ± 0.004	9	0.17 ± 0.007	5	0.962
Kidney; Right	0.14 ± 0.005	8	0.15 ± 0.004	9	0.15 ± 0.006	5	0.229
Pancreas	0.12 ± 0.01	8	0.13 ± 0.006	9	0.13 ± 0.009	5	0.701
Spleen	0.06 ± 0.002	8	0.06 ± 0.003	9	0.07 ±0.007	5	0.212
Brain	0.45 ± 0.004	8	0.45 ± 0.005	9	0.44 ± 0.002	5	0.729

4.3.2 Serological analysis

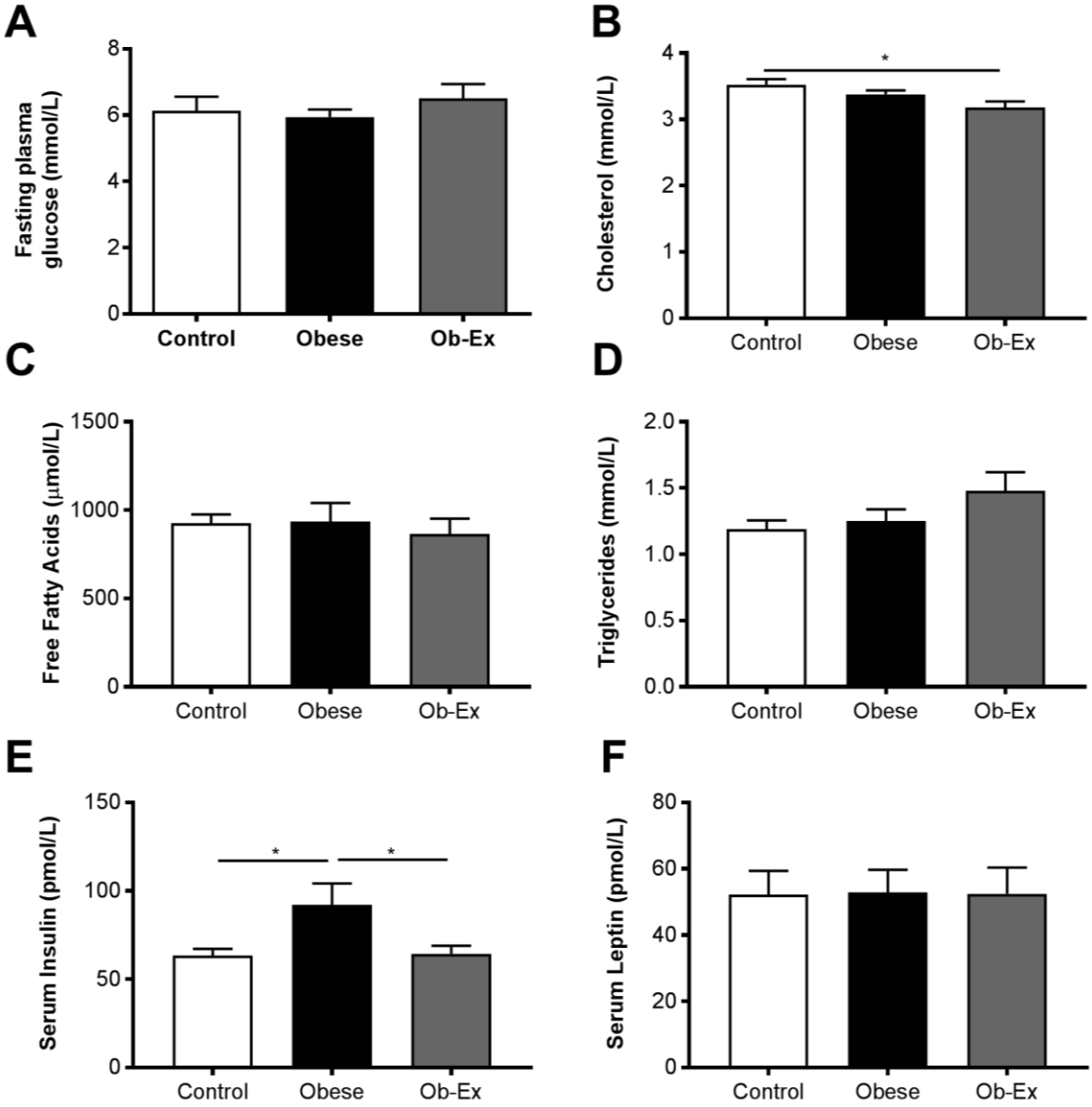


FIGURE 4.4: Serological analysis in 16 hour fasted serum. Methods for serum analysis is described in Section 2.3. Control *n*= 9, Obese *n*= 6 and Ob-Ex *n*= 9. Except for cholesterol (***2 outliers*** were detected by Grubbs test) Control ***n= 8***, Obese ***n= 5***, Ob-Ex *n*= 9 and Leptin (***1 outlier*** detected) Control *n*= 9, Obese ***n= 5*** and Ob-Ex *n*= 9. Insulin ELISA was performed by Dr Denise Fernandez-Twinn and previously published (Fernandez-Twinn *et al.*, 2017). One-way ANOVA with Bonferroni post-hoc test; * $p< 0.05$.

There were no significant differences in fasting plasma glucose when measured from tail blood before the *post mortem* (Figure 4.4A). Total cholesterol was decreased ($p< 0.05$) between the offspring of control and obese-exercised dams (Figure 4.4B). Serum triglycerides and free fatty acids were not different between the offspring groups (Figure 4.4C+D). Serum insulin was increased in offspring of obese dams (compared to control) and this increase was

prevented with a maternal exercise intervention (Figure 4.4E). Serum leptin was unchanged between groups (Figure 4.4F).

4.3.3 Cardiac phenotype

Pathological cardiac hypertrophy

Offspring of obese dams had increased heart weight when expressed as an absolute weight (Figure 4.5A) and when expressed relative to bodyweight (Figure 4.5B) (as bodyweight was not different between the groups - see Table 4.2). This increase was prevented by the maternal exercise intervention.

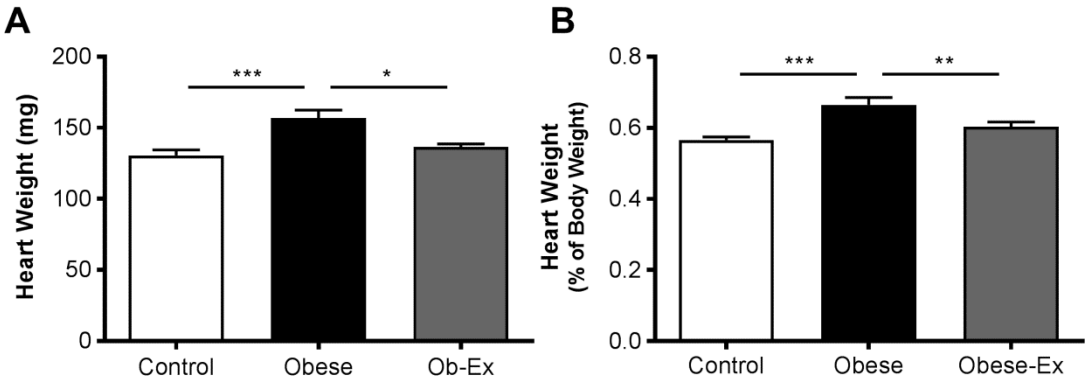


FIGURE 4.5: Heart weights at *post mortem*. Heart weight was measured by weighing wet tissue weight at *post mortem* post 16 hour fast. A) Heart weight B) Heart weight expressed relative to bodyweight. Hearts collected by Dr Sarah Carr. Control n= 11, Obese n= 9 and Ob-Ex n= 10. One-way ANOVA with Bonferroni post-hoc test; * p< 0.05, ** p< 0.01 and *** p< 0.001.

Cardiac hypertrophy was further assessed by measurement of cardiomyocyte cell area in the mid-cardiac sections of the hearts (representative images in Figure 4.6A). A mean average was calculated by averaging all cell areas measured for each individual animal, then the six animals analysed were averaged to give an overall mean cardiomyocyte cell area of that group. Mean cardiomyocyte cell area was increased in offspring of obese dams (Figure 4.6B). Frequency distribution of cardiomyocyte cell areas showed a rightward shift, indicative of a higher proportion of larger cells in the offspring of obese dams, thereby confirming hypertrophy (Figure 4.6C). These parameters were normalized in offspring of obese-exercised dams. Hierarchical linear model analysis showed that the p value for the effect of maternal diet to increase cell size was p< 0.001, and for exercise to prevent this increase was p< 0.001. This specialised statistical analysis was kindly performed by Dr Catherine Aiken.

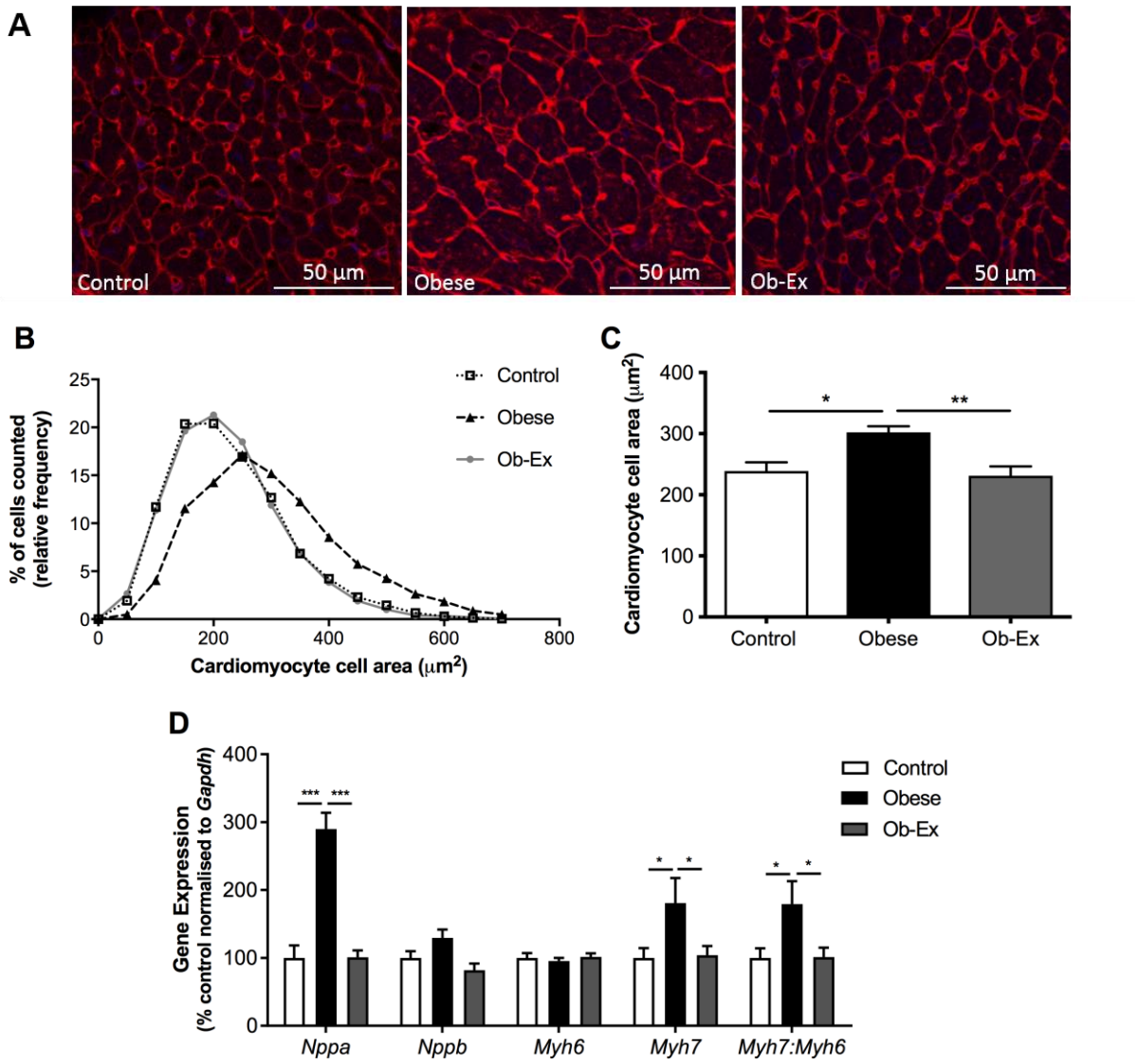


FIGURE 4.6: Pathological cardiac hypertrophy markers in male offspring at 8 weeks of age. Representative images of wheat germ agglutinin stained mid-cardiac sections (analysed manually by CellD). B) Mean cardiomyocyte cell area. C) Frequency distribution of cardiomyocyte cell area. *n*= 6 hearts per group with ~7000 cells analysed per group. Hearts for WGA analysis were collected by Dr Heather Blackmore. D) Ventricular fetal gene expression. *Gapdh* was the housekeeper and was unchanged between groups. Control *n*= 9, Obese *n*= 7 and Ob-Ex *n*= 9. One-way ANOVA with Bonferroni post-hoc test; * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$. *Nppa*- atrial natriuretic peptide, *Nppb*- brain natriuretic peptide, *Myh7*- β-MHC and *Myh6*- α-MHC.

Atrial natriuretic peptide (*Nppa)* expression was increased approximately three-fold in the hypertrophic hearts of the offspring of obese dams; this expression was normalized in the offspring of the intervention group (Figure 4.6D). Furthermore the *Myh7: Myh6* ratio was increased in the hearts of offspring exposed to maternal obesity, which was driven by increased *Myh7* rather than a change in *Myh6* (Figure 4.6D). This was also normalized in the intervention group (Figure 4.6D).

Cardiac fibrosis

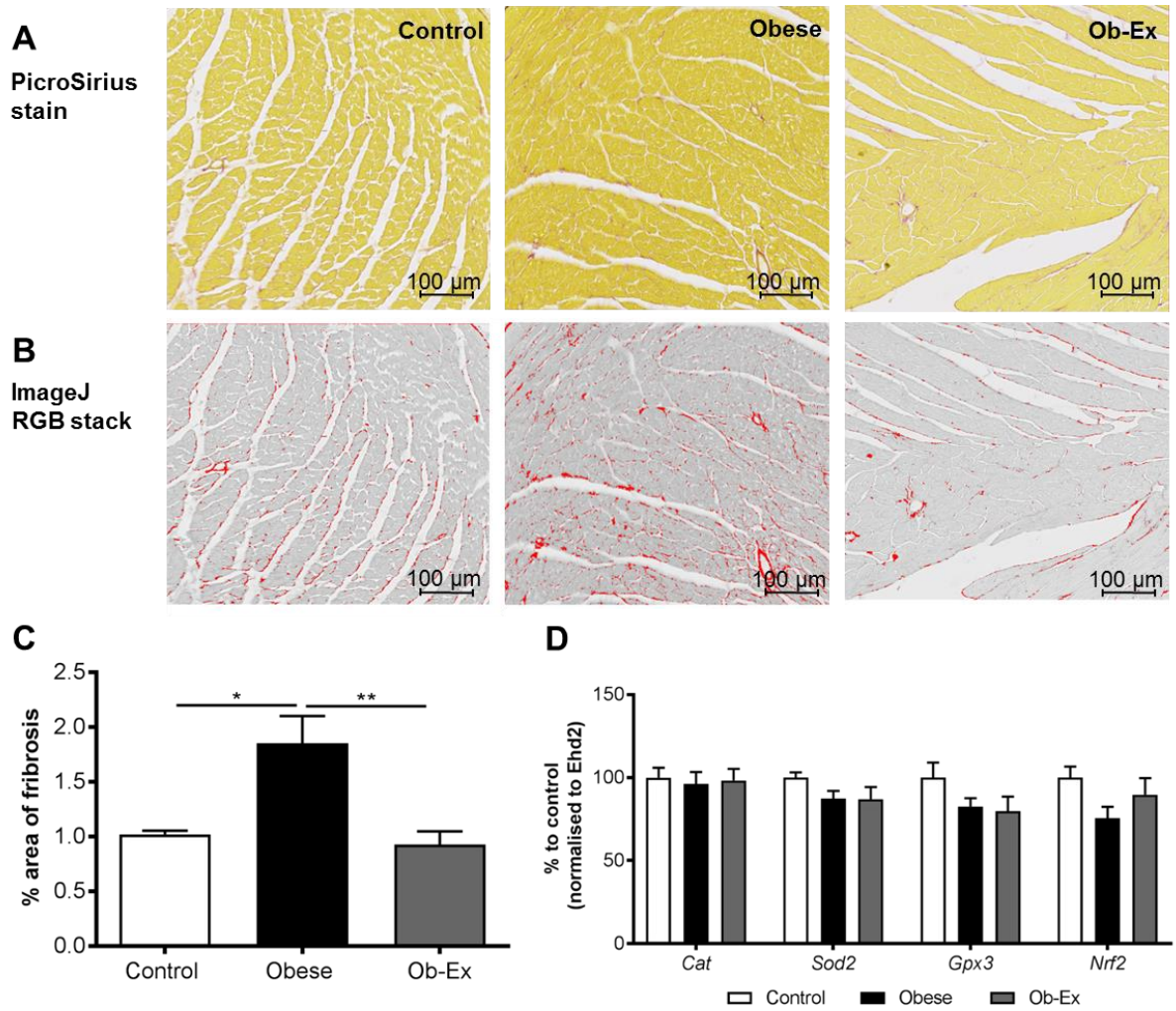


FIGURE 4.7: Assessment of cardiac fibrosis and oxidative stress. A) Representative PicroSirius red stained heart sections. B) Images converted into RGB stack in ImageJ for analysis. C) % area of fibrosis (% area of red stain) was assessed in three groups. Hearts sections for staining provided by Dr Heather Blackmore and Dr Sarah Carr. Control *n*= 7, Obese *n*= 8 and Ob-Ex *n*= 5. One-way ANOVA with Bonferroni post-hoc test; * $p< 0.05$ and ** $p< 0.01$. D) Expression of antioxidant defence genes: *Cat*- Catalase, *Sod2*- Superoxide dismutase 2, *Gpx3*- Glutathione peroxidase and *Nrf2*- Nuclear factor erythroid 2–related factor 2. Normalised to housekeeper *Ehd2*- EH Domain Containing 2. Control *n*= 8, Obese *n*= 8 and Ob-Ex *n*= 6.

Mid-cardiac heart sections were assessed for fibrosis using a PicroSirius red stain. Representative images from all three groups can be seen in Figure 4.7A. Total fibrosis (% area stained) was increased in the offspring of obese dams compared to both offspring of control and obese-exercised dams (Figure 4.7C). This was a modest increase of less than two-fold and was not accompanied by any changes in the mRNA expression of key oxidative stress response genes such as Catalase (*Cat*) or Superoxide dismutase 2 (*Sod2*) (Figure 4.7D).

Offspring blood pressure

BP was assessed in the offspring through non-invasive tail cuff plethysmography. Exposure to maternal obesity causes elevated SBP in the male offspring at eight weeks of age (Figure 4.8A). This occurred without any change in pulse rate (Figure 4.8B). Maternal exercise intervention in an obese pregnancy did not prevent the increase in SBP. The width of ascending aorta was measured during *in vivo* echocardiographic analysis using EKV^{TM} images. The diameter at systole was increased in offspring of obese dams (Figure 4.8C). The diameter at diastole was unaltered (data not shown), but the calculated difference in width between systole and diastole was increased in offspring from obese dams (Figure 4.8D).

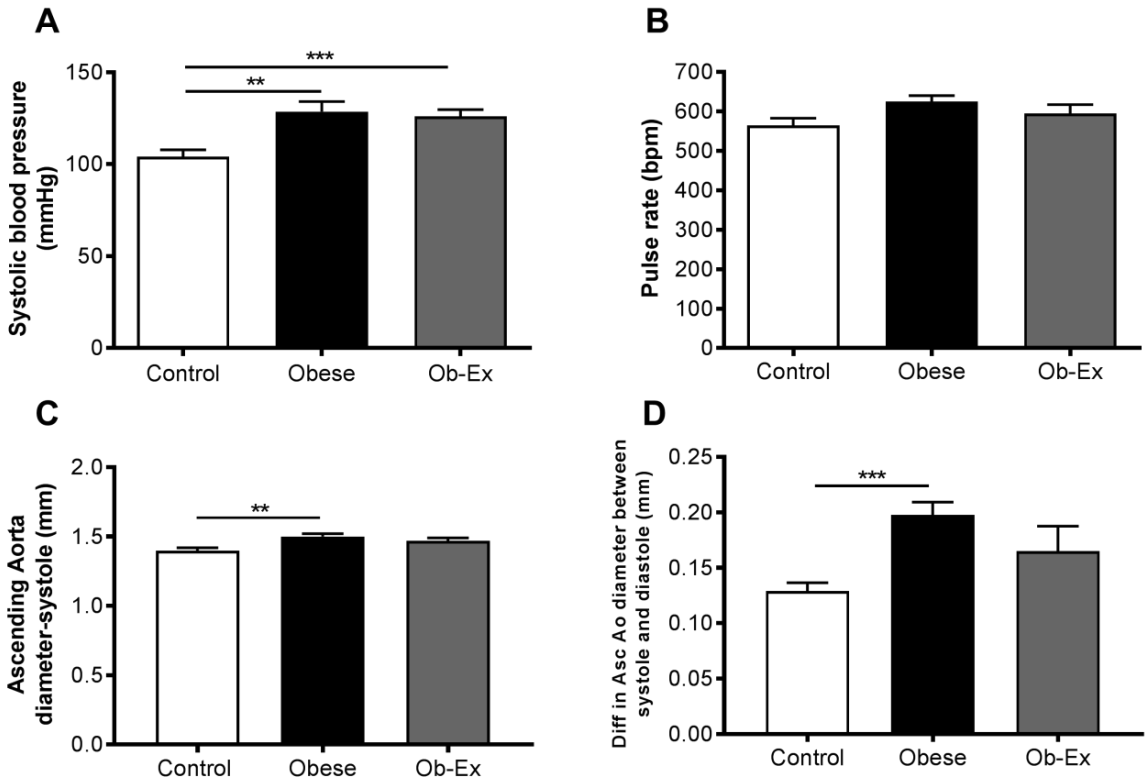


FIGURE 4.8: Offspring BP and aortic diameter. A) BP measurements were carried out at the start of dark (active cycle) at the same time (16:00) across three consecutive days. Measurements were taken from the 3rd day when variation between measurements was lowest. This is fully described in Section 2.4. B) Pulse rate (bpm). Control *n*= 16, Obese *n*= 14 and Ob-Ex *n*= 10. C) Asc Ao diameter measured 1 mm from aortic valve in EKV^{TM} images at end-systole. Control *n*= 13, Obese *n*= 10 and Ob-Ex *n*= 6. D) Difference in Asc Ao diameter between end-systole and end-diastole. Control *n*= 10, Obese *n*= 9 and Ob-Ex *n*= 4. One-way ANOVA with Bonferroni post-hoc test; ** $p< 0.01$ and *** $p< 0.001$.

Offspring of obese-exercised dams had increased SBP but no significant difference is seen in the aortic width diameters when compared to either the offspring from control or obese dams.

The measured values were in between the values for the two other groups. The lack of significance may reflect the fact that this group is relatively underpowered compared to other groups.

In vivo cardiac function

Left ventricular end-systolic volume (ESV) was elevated in male offspring of obese dams (Figure 4.9A) but there were no differences in the volume at diastole (Table 3). There was no significant effect on stroke volume (Figure 4.9B), however there was an accompanying reduction in ejection fraction (Figure 4.9C). This contributed to a significant overall effect of maternal obesity on reducing cardiac output in the offspring (Figure 4.9D) despite no differences in heart rate (Table 4.3). Furthermore, diameter of the ventricular lumen was increased at the end of systole in offspring of obese dams suggesting impaired cardiac contractility (Figure 4.9E). This was confirmed by a reduction in ventricular fractional shortening in the offspring of obese dams (Figure 4.9F).

Offspring of the obese-exercised group did not have increased left ventricular ESV and end-systolic diameter, and the functional parameters were restored to values matching offspring of control dams (Figure 4.9A-F). Structurally, the diameter of the IVS and LVPW at systole in the intervention group was increased compared to the offspring of non-exercised obese dams. This resulted in an increased systolic wall: lumen width ratio in the offspring of the obese-exercised dams (Table 4.3).

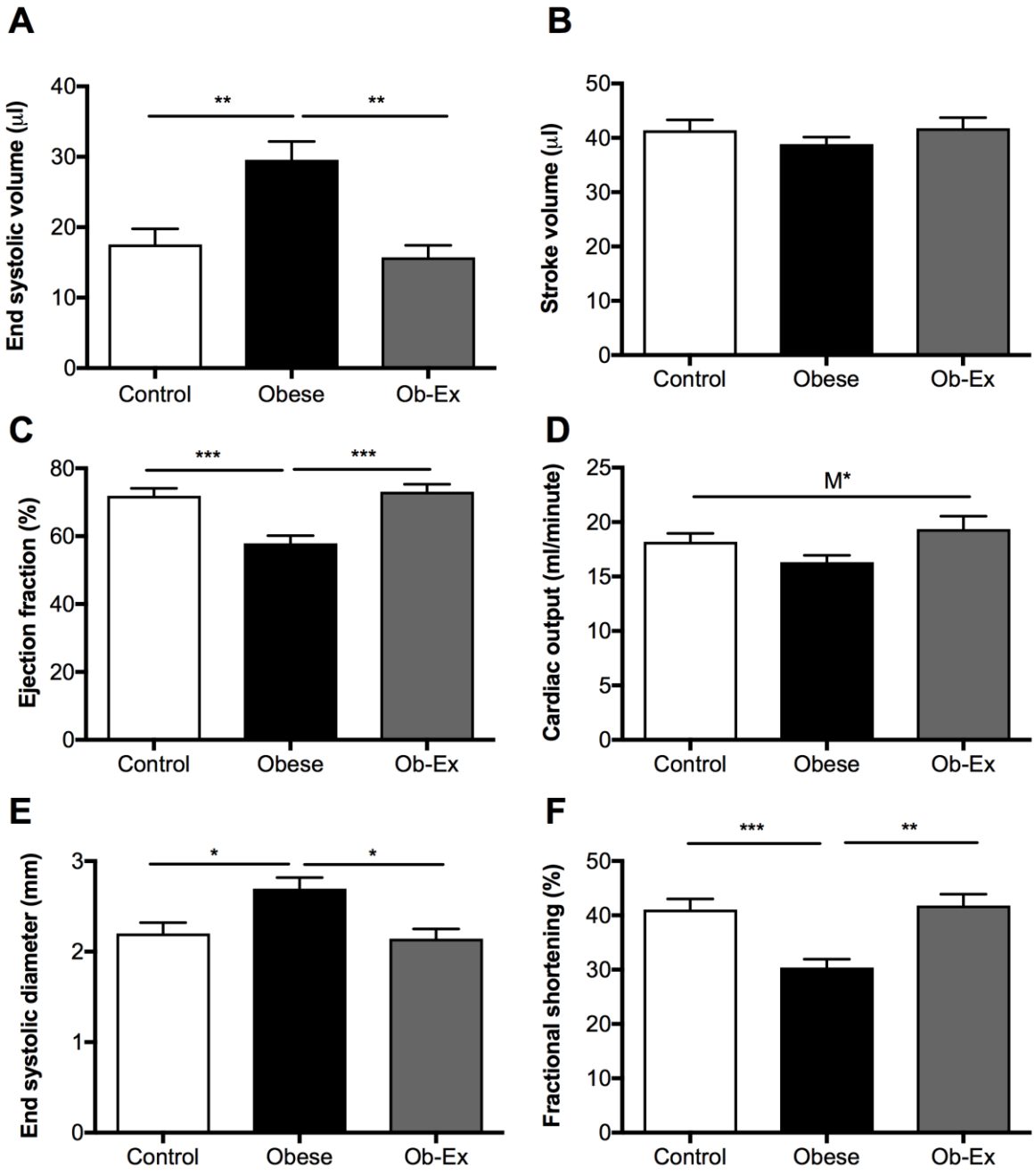


FIGURE 4.9: Functional parameters assessed by *in vivo* echocardiography. A)-F) Systolic function and contractility measured by echocardiography. Parameters analysed by LV trace tool in M-mode. Control *n*= 16 and Obese *n*= 16 and Ob-Ex *n*= 7. One-way ANOVA where M*- overall effect of maternal lifestyle $p< 0.05$. Post-hoc Bonferroni testing; * $p< 0.05$ ** $p< 0.01$, *** $p< 0.001$. Echocardiography analysis was performed in collaboration with Dr Denise Fernandez Twinn, Dr Heather Blackmore and Dr Sarah Carr.

TABLE 4.3: *In vivo* echocardiographic parameters. Data is presented mean ± SEM. Control *n*= 16, Obese *n*= 16 and Ob-Ex *n*= 7. *P* values calculated by one-way ANOVA. Letters represent differences between specified groups by Bonferroni post-hoc test ($p< 0.05$); [a] Control vs. Obese and [c] Obese vs. Ob-Ex.

	Control	*Obese*	*Ob-Ex*	*p value*
End diastolic volume (mm^3)	58.9 ± 3.9	68.4 ± 3.0	59.2 ± 3.0	0.097
End diastolic diameter (mm)	3.70 ± 0.10	3.95 ± 0.08	3.72 ± 0.08	0.092
Heart Rate (bpm)	443 ± 12	421 ± 11	446 ± 17	0.291
IVS; diastole (mm)	0.924 ± 0.04	0.869 ± 0.05	0.957 ± 0.05	0.440
LVID; diastole (mm)	3.65 ± 0.10	3.92 ± 0.07	3.71 ± 0.07	0.061
LVPW; diastole (mm)	0.85 ± 0.04	0.82 ± 0.03	0.89 ± 0.07	0.620
Wall:Lumen ratio; diastole	0.24 ± 0.01	0.21 ± 0.01	0.24 ± 0.02	0.279
IVS; systole (mm)	1.37 ± 0.05	1.25 ± 0.05	1.51 ± 0.09[c]	**0.019**
LVID; systole (mm)	2.32 ± 0.12	2.82 ± 0.10[ac]	2.24 ± 0.14	**0.003**
LVPW; systole (mm)	1.34 ± 0.05	1.18 ± 0.05	1.42 ± 0.11[c]	**0.030**
Wall:Lumen ratio; systole	0.57 ± 0.04	0.43 ± 0.03	0.65 ± 0.08[c]	**0.007**
LV mass (mg)	90.2 ± 3.6	98.0 ± 3.9	97.6 ± 10.0	0.453

† LV= Left ventricle, IVS= Interventricular septum, LVID= LV internal diameter and LVPW= LV posterior wall.

The expression of contractile machinery proteins involved in Ca^{2+} handling pathways were assessed by western blot. Although maternal obesity did not have any effect on the levels of any of the key cardiac contractile machinery proteins (compared to the Control group), levels of Troponin I and Tropomyosin were increased over two-fold in the ventricular tissue from the offspring of obese-exercised dams compared to offspring of control and obese groups (Figure 4.10A+D). Levels of activated phos-Troponin I (Ser23/24) were also increased, which

reflected an increase in the phos:total Troponin I ratio compared to the other two groups (Figure 4.10B+C). Furthermore, SERCA2, a protein involved in the recycling of Ca^{2+}, was also increased in the adult male offspring of obese-exercised dams. (Figure 4.10E).

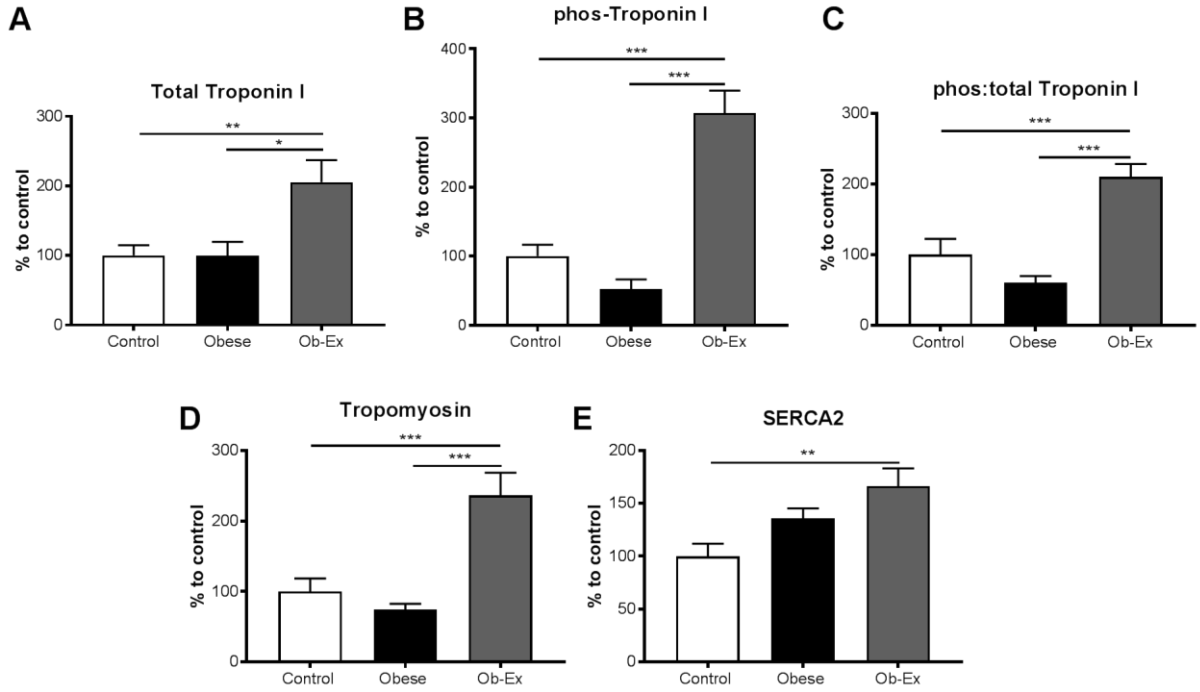


FIGURE 4.10: Expression of key contractile machinery proteins. Expression level assessed by western blotting. Control *n*= 9, Obese *n*= 8 and Ob-Ex *n*= 8. One-way ANOVA with Bonferroni post-hoc test; * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

4.4 Discussion

The aim of this chapter was to assess the cardiovascular outcomes of maternal obesity and then to assess the potential protective effects of the maternal exercise intervention. Pathological cardiac hypertrophy and *ex vivo* cardiac dysfunction has previously been demonstrated in male offspring from our model (Blackmore *et al.*, 2014) and this cardiovascular phenotype has been further investigated in this chapter. *In vivo* cardiac dysfunction and pathological cardiac hypertrophy accompanied increased SBP in offspring of obese dams when compared to offspring of lean dams (Control diet-fed dams). The impact of maternal exercise on the poor cardiovascular health of the offspring of obese dams was also assessed and showed protective effects by the reversal of cardiac hypertrophy and dysfunction while failing to address offspring elevated SBP. This shows there has been a successful implementation of an intervention in the mother that has long-term impact on offspring cardiovascular outcomes. This information could be used to support the need for long-term

follow up in the offspring of already established human intervention studies. Just a small improvement in offspring risk could have important implications for the future burden of CVD worldwide.

4.4.1 Offspring body composition and serum analysis

It was a particular strength to be able to assess body composition longitudinally until eight weeks old. The significant effects of age on bodyweight and body composition track the male mouse as it matures from a juvenile to an adult. There was an effect of maternal exercise on offspring fat mass, with multiple comparison testing showing differences between offspring of control and obese dams at seven and eight weeks of age. This occurred with no accompanying changes in bodyweight or lean mass, despite the animals being fed the same control chow diet (RM1). When the TDNMR fat mass at eight weeks of age was considered separately, there were no statistical differences similarly there were no measurable differences in the weighed fat depots. This difference in fat mass was not great enough to cause any changes in serum leptin.

An increase in serum insulin was observed in offspring of obese dams at eight weeks of age, this has been shown in previous cohorts (Fernandez-Twinn *et al.*, 2012). This occurred without a difference in body composition between the offspring at eight weeks of age. Body fat percentage in adult males from Indonesia has been shown to be a good predictor of insulin resistance (Boy Kurniawan *et al.*, 2018). We have shown previously that there was an exercise-induced improvement in insulin sensitivity in the obese dams during pregnancy, this improvement is mirrored in the eight week old male offspring (Fernandez-Twinn *et al.*, 2017). Exercise improves insulin sensitivity (Gollisch *et al.*, 2009), and despite the offspring not exercising themselves (intervention was in the mother only) they have a normalisation in their serum insulin. Normalisation of serum insulin by maternal exercise has also been shown in other studies both in offspring from obese (Bae-Gartz *et al.*, 2015) and lean mouse dams (Carter *et al.*, 2013). Through correlations an association has previously been shown between maternal and offspring serum insulin (Fernandez-Twinn *et al.*, 2017). The data in this thesis showing that a maternal exercise intervention that prevents maternal hyperinsulinemia also prevents offspring hyperinsulinemia supports the hypothesis that this is a causal relationship. The mechanisms by which maternal hyperinsulinemia causes offspring insulin resistance and potentially cardiac dysfunction are outside of the thesis aims and are not currently known.

4.4.2 Maternal exercise prevented pathological cardiac hypertrophy

Offspring of obese dams had pathological cardiac hypertrophy at eight weeks of age, in fact this has previously been shown to occur from as early as three weeks of age (Blackmore *et al.*, 2014). This increase in heart weight appears to be the result of cardiac remodelling, as indicated by previous cardiac stereology data that showed increases in LV volume at the same age (Blackmore *et al.*, 2014). Cardiomyocyte cell area was assessed in the LV, and this demonstrated cell specific hypertrophy that could account for the increased heart weight and LV volume. A hierarchal linear model was also applied for a stringent assessment of changes in the frequency distribution of cardiomyocyte area. This is a powerful statistical analysis that takes into account the fact that cell measurements were obtained from multiple sections of the heart of each individual animal within the groups, and therefore cannot be treated as fully independent. Consequently, it can be concluded not only, that the mean cardiomyocyte area is increased in the offspring from obese dams, but that there was a significant rightward shift in the frequency distribution indicating a higher proportion of larger cells.

In the heart during haemodynamic overload, there is re-expression of the fetal genes *Nppa* and *Myh7*, both of which are highly conserved markers of ventricular pathological hypertrophy (Pandya and Smithies, 2011; Chien *et al.*, 2016). This re-expression can lead to decompensation and heart failure (Distefano and Sciacca, 2012). *Myh6* (α-MHC, adult isoform) and *Myh7* (β-MHC, fetal isoform) are two functionally distinct cardiac isoforms. In postnatal life, α-MHC completely replaces β-MHC in the ventricles, becoming the dominant isoform (>90%) in the adolescent mouse (Lyons *et al.*, 1990). *Myh7* has a lower sliding velocity, therefore an increased ratio of *Myh7: Myh6* will negatively impact cardiac function (Holubarsch *et al.*, 1985). This can be explained as the period of time available for ejection is finite, therefore any decrease in sliding velocity reduces the rate of volume ejection, leaving more blood behind at the end of systole (increased ESV) and decreasing stroke volume. Genetic alteration of MHC isoforms indicated that even a small shift in expression of β-MHC (12%) resulted in physiologically significant changes in cardiac contractility (Tardiff *et al.*, 2000). Maternal obesity caused the up-regulation of *Nppa* and *Myh7* in cardiac tissue of adult male offspring, concurrent with other markers of pathological cardiac hypertrophy.

It is striking that the maternal exercise intervention was able to reverse all measured parameters of pathological cardiac hypertrophy in the offspring of obese dams; including heart weight, cardiomyocyte cell area and the re-expression of fetal genes. Reversal of LV

hypertrophy will have important implications for that individual's future cardiovascular health, as reversal has been associated with a reduction in arrhythmias, improved diastolic function, preserved systolic function and increased coronary reserve (Dahlof, 1992). Many studies have shown that it is possible to reverse hypertrophy through the use of anti-hypertensive therapies, and that this improves cardiac performance, lowers CVD morbidity and mortality risk independent of the reduction in BP. Therefore, the prevention of cardiac hypertrophy in the offspring of obese dams with the maternal exercise intervention is a very important outcome that has improved their cardiovascular health.

Cardiac hypertrophy was accompanied by increased interstitial fibrosis in the mid-cardiac section of the heart and is an indication of cardiac remodelling, in particular in the form of alterations in the extracellular matrix. The measured increase in fibrosis was mild and was not accompanied with changes in mRNA expression of genes critical for the oxidative stress response. There is a need, in the future, to measure these antioxidant defence genes at the protein level to fully assess if there are any changes in the oxidative stress response. However, fibrosis can disrupt myocardial architecture, cause cardiac dysfunction and promote the progression of heart disease (Segura, Frazier and Buja, 2014). There was no difference in fibrosis between the hearts from offspring of control dams and offspring of obese-exercised dams, this is a positive finding as therapies that reduce the severity of fibrosis were effective at improving outcomes in heart disease (Schelbert *et al.*, 2014).

One focus of this thesis was to address the cause of the observed cardiac hypertrophy. One potential mechanism that we addressed was the possibility that the offspring were hypertensive, as this has been seen in a similar rodent model of maternal overnutrition at an older age (Samuelsson *et al.*, 2010). We measured BP non-invasively in the offspring and there was an increase after exposure to maternal obesity, however this was maintained with maternal exercise whereas hypertrophy was not. This mismatch in these offspring outcomes in the intervention group indicates that offspring hypertension cannot be the only driver in the development of cardiac hypertrophy. A study in hypertensive children showed that the relationship between LV hypertrophy and BP explains only 25–30% of the variation in LV mass (Sorof *et al.*, 2002), therefore suggesting another mechanism is at play. One of these could be the growth effect of insulin which is described in the next section.

4.4.3 Possible mechanisms for the reversal of cardiac hypertrophy by maternal exercise

Previous work in our laboratory has shown that offspring hyperinsulinemia is associated with the programming of cardiac hypertrophy in offspring of obese dams (Fernandez-Twinn *et al*., 2012). Data from this thesis supports this conclusion, as offspring of obese-exercised dams did not have cardiac hypertrophy and serum analysis showed no hyperinsulinemia, unlike offspring of obese dams who have both parameters. This previous study showed an association with increased serum insulin and an increase in PI3K and MAPK signalling pathways in the offspring heart tissue. PI3K signalling has commonly been associated with physiological cardiac hypertrophy (Ma *et al.*, 2013), however constitutive activation seen in a transgenic mouse with AKT overexpression (a protein critical in PI3K pathway) can be damaging and led to cardiac hypertrophy accompanied by interstitial fibrosis and cardiac dysfunction (Shiojima *et al.*, 2005).

Chronic hyperinsulinemia experimentally induced in rats for seven weeks increased LV mass and wall thickness while also reducing stroke volume and cardiac output (Samuelsson *et al.*, 2006). Similarly, the reduction of plasma insulin ameliorates systolic dysfunction induced by a TAC model of pressure overload, with insulin treatment having the opposite effect of enhancing cardiac hypertrophy and decreasing systolic function (Shimizu, Ippei, Komuro, 2010). In hypertensive patients the increased LV mass is associated with increased fasting plasma insulin levels and decreased whole body glucose disposal (Paolisso *et al.*, 1995).

Oxidative stress could also be a cause of the observed cardiac hypertrophy and accompanying interstitial fibrosis, with other models of developmental programming implicating oxidative stress as critical in the development of CVD (Wang *et al.*, 2010; Giussani *et al.*, 2012). Offspring of obese mothers who showed increased PI3K and MAPK signalling (as previously described), also had enhanced lipid peroxidation and impaired antioxidant capacity (Fernandez-Twinn *et al.*, 2012). In this thesis the mRNA expression of key players in antioxidant defence was assessed but did not show any differences, however changes in expression of these genes can often occur at the post-transcriptional level and protein expression of these loci has not been explored. Elevated gene expression of oxidative stress markers is known to occur in E19 hearts (see Appendix AP3) although this does not result in changes in lipid peroxidation (see Section 6.3.6).

Since offspring from obese-exercised dams were hypertensive without hypertrophy, the mechanism of pressure overload triggering cardiac hypertrophy has been placed in doubt. The

cardiac hypertrophy cannot be a consequence of hypertension but must instead be programmed through a discordant mechanism that is modifiable through the maternal exercise intervention. The fact that hypertension was not corrected in the offspring exposed to maternal exercise intervention leads us to assess maternal factors not corrected by the intervention as a potential mechanism.

4.4.4 Possible programming factors for offspring hypertension

Increased fetal exposure to glucocorticoids is thought to be one mechanism that is responsible for programming hypertension (Wintour *et al.*, 2003; Dagan *et al.*, 2007), however little information is available on other potential mediators; such as maternal-to-fetal hormones and growth factors. One factor which has gained more interest recently is maternal leptin, which we (Fernandez-Twinn *et al.*, 2017), and others (Vega *et al.*, 2016), have shown is not corrected by a maternal exercise intervention.

Leptin has a neurotrophic role in the development of critical neuronal projections in key hypothalamic nuclei such as the arcuate nucleus of the hypothalamus (ARC) (Bouret, Draper and Simerly, 2004). Rodents have a characteristic neonatal leptin surge during the first two weeks of postnatal life which is also a critical developmental window for neurocircuits in the ARC to be established (Ahima, Prabakaran and Flier, 1998). An elevated leptin surge has been shown in neonates exposed to gestational hypoxia (12% E15-E19) resulting in hyperphagia, this could be caused by increased basal ARC activation and altered ARC projections to the paraventricular nucleus, however the authors of this study do not assess effects on BP (Vargas *et al.*, 2017). It has been shown that inducing experimental hyperleptinemia in neonatal rat pups from postnatal day 9 to 15 causes increased BP in adulthood (Samuelsson *et al.*, 2016) and programs long-term renal structural and functional damage, through renal sympathetic nerve activation (Oosterhuis *et al.*, 2017). It has also been suggested that an exaggerated neonatal leptin surge stimulates hypothalamic melanocortin signalling leading to altered sympathetic tone (Samuelsson *et al.*, 2010). The lack of effectiveness of the exercise intervention to prevent offspring hypertension therefore may be related to the maintained hyperleptinemia in the obese-exercised dams.

The critical window of prenatal exposure for the programming of adult hypertension has been identified in the rat. Maternal calorie restriction programmes hypertension in male and female offspring when imposed throughout the whole of gestation, or only during the second half of gestation, but has no effect on offspring adult BP when imposed during only the first half of

pregnancy (Vehaskari, Aviles and Manning, 2001). Since our exercise protocol continues to E17 and their ability to run declines during gestation; the greatest impact of our intervention may be in the early part of pregnancy and this may explain why we have not been able to prevent the programming of hypertension in our offspring. This highlights the importance of using an intervention at different critical time windows to dissect out the different mechanisms that ultimately control the complex changes to environmental cues that the offspring experiences during development.

What is striking is that the critical window of susceptibility to altering BP coincides with kidney development in the late stages of pregnancy, and it is therefore likely that altered renal morphology could be responsible. Decreased glomerular density results in changes in tubule formation and this will impact on the development of ion transporters. Models of maternal diabetes and maternal low protein diet have shown offspring have increased tubular ion channel expression and ion transport, these offspring also have increased BP suggesting the subsequent reduced salt excretion could be the cause of the increased BP (Manning *et al.*, 2002; Nehiri *et al.*, 2008). Decreased nephron number was also demonstrated in offspring exposed to antenatal protein restriction and this resulted in reduced glomerular filtration rate (Vehaskari and Woods, 2005). Equally, it could be that morphological changes in the kidney could be a consequence and not a cause of the accompanying hypertension. In the same model used in this thesis, eight week old offspring have glomerular hypertrophy (Pinnock 2018, personal communication), this is indicative of increased glomerular pressure possibly caused by the increased BP (D'Agati *et al.*, 2016).

In humans, persistent hypertension can cause significant enlargement of the aortic root width, compared to age and sex-matched populations of similar body size (Kim *et al.*, 1996). The ascending aorta widens upon contraction, as it stretches to accommodate the blood ejected out from the LV. We observed a greater increase in aortic width following ventricular contraction, consistent with our functional data that shows adult offspring of obese dams experiencing increased haemodynamic stress and resulting in cardiac dysfunction caused by an increased SBP. The difference in width at systole and at diastole is also greater, consistent with increased stretch, as a greater force is needed to eject blood out of the LV against the increased BP.

Limitations with tail cuff plethysmography method

Tail cuff was chosen over telemetry for technical and scientific reasons. Firstly, telemetry is recommended in mice heavier than 25 g (Kurtz *et al.*, 2005; Huetteman and Bogie, 2009), and our experimental animals had not reached this weight by the time of BP measurement. Telemetry probe implantation requires specific technical expertise and the weight of the probe can often compromise animal welfare. Insertion of the probe involves permanent ligation of an artery which can cause circulation perturbations, and also requires the animal to be singly housed which is considered a stressful experience in rodents due to the social isolation (Bartolomucci *et al.*, 2003; Polycarpou *et al.*, 2016). Secondly, as echocardiography and BP were performed on the same mouse, we wanted to avoid the animal having to be anaesthetized on more than one occasion.

Many publications have assessed the differences between the tail cuff and telemetry methods, including the effect of stress. The physiology of the stress response encompasses the whole body and therefore is an important consideration in data interpretation and experimental design. One study showed a slight underestimation of tail cuff BP measurements during restraint compared to telemetry. However, when compared to non-disturbed telemetry mice the readings were very comparable. A study using the same maternal diet-induced obesity model has previously shown hypertension in older offspring (12 weeks of age) using a telemetry method (Samuelsson *et al.*, 2008); with tail cuff we were able to recapitulate this outcome in a younger males. We have adapted our interpretation of the data with a sensitivity and awareness of the limitations of the technique. Tail cuff measurements are not well suited for measuring diastolic measurements (Reddy *et al.*, 2003), therefore we have not included this data for analysis. We also endeavoured to minimise stress to the animal by conditioning the animal to restraint over three days, keeping the experimenter the same and performing it at start of their active cycle so as not to abruptly awaken them from sleep.

4.4.5 Mechanisms underlying the improved offspring cardiac function by maternal exercise intervention

In the previous section we have shown that the hearts of offspring of obese dams are under increased afterload (force against the heart contracts) caused by the increased SBP. They will have to overcome a greater pressure to successfully eject blood out of the ventricle. If preload and inotropy remain constant, this change will result in impaired cardiac function. Increased afterload can result in an increase in ESV with a subsequent reduction in stroke volume. The

force-velocity relationship states that increased afterload will reduce the velocity of muscle fibre shortening; thereby reducing the velocity the ventricle can eject the blood. Less blood is able to be ejected from the heart and with more left behind, the ESV increases (Tarazi and Levy, 1982). The afterload in the offspring of obese dams was great enough to adversely impact on LV systolic function and myocardial contractility, as ESV was increased, and fractional shortening and ejection fraction were reduced. This suggests that adult offspring of obese mothers cannot maintain cardiac output under this haemodynamic stress, a pre-eminent feature of heart failure (Weber, Janicki and Maskin, 1985).

As demonstrated, offspring of obese dams were hypertensive irrespective of the exercise intervention, therefore the increased afterload should also be present in the offspring of obese-exercised dams, and yet their functional parameters were normalised. Restored ejection fraction, cardiac output and fractional shortening were seen with maternal exercise intervention. It is possible that there has been a compensatory increase in contractility to this haemodynamic stress. Increased wall contractility is indicated through the increased ratio of wall: lumen widths at systole compared to offspring of lean dams. This is a compensatory response that the offspring of obese dams perhaps could not mount or maintain, and this suggests a lower adaptive ability to high workload conditions. After a high workload challenge it was shown that there was a failure to maintain adequate cardiac workload in the *ex vivo* fetal sheep hearts of offspring exposed to maternal overnutrition (Wang *et al.*, 2010). The pathological cardiac hypertrophy present in these animals could be the cause of their lack of ability to respond. Alternatively, hypertrophy could be a consequence and represent the failed adaptive response to lower wall tension and enhance contractility. This uncertainty for the ability of the heart to adapt to changing workloads is a concern in a young animal; as this diminished cardiac reserve could mean these animals could go on to develop more significant cardiac dysfunction with age.

Modulation of Ca^{2+} pathways is critical for the heart to adapt to changing workloads (Chung *et al.*, 2016). Key proteins in these pathways were elevated in ventricular tissue from offspring of obese-exercised mothers compared to offspring of both control and non-exercised obese mothers, this supports a role for increased inotropy as a potential driver of their improved cardiac function. Sympathetic activation occurs through the β1-adrenergic receptor which activates Protein Kinase A and the phosphorylation of Troponin I, which decreases the Ca^{2+} sensitivity of Troponin C causing an increased off-rate of Ca^{2+}, allowing accelerated

cardiac relaxation (Bodor *et al.*, 1997). An up-regulation of proteins in this pathway is indicative of an enhanced sympathetic response. Furthermore, elevated SERCA2 expression would allow Ca^{2+} to be recycled back to the sarcoplasmic reticulum facilitating efficient future cardiac contractions. Total Troponin I and Tropomyosin in the myocardium were also increased and could further explain the improved cardiac function.

Although we have shown that cardiac dysfunction is normalized in offspring of obese-exercised dams, it is not yet clear if these changes are beneficial to cardiac health long-term and whether the necessary adaptation to combat increased afterload can be maintained. Therefore, future studies should address this and consider an ageing component, it will be equally important to determine if there is further deterioration in the offspring of obese dams without intervention.

4.4.6 Discordant programming pathways

These findings highlight discordance between the mechanisms through which maternal obesity during pregnancy mediates the programming effects on offspring cardiac dysfunction, compared to those leading to hypertension. Our data suggest that cardiac hypertrophy measured in offspring of obese dams is not a consequence of hypertension but is heavily associated with the observed cardiac dysfunction. These outcomes could be programmed by maternal insulin, as we have successfully targeted maternal hyperinsulinemia in the exercise intervention. The fact that hypertension was not corrected in the offspring exposed to maternal exercise intervention leads us to consider maternal factors not corrected by the intervention, with maternal leptin discussed as a potential mechanism.

4.4.7 Conclusions

An intervention that is able to make a significant impact while only being implemented during pregnancy is important from a translational perspective. Pregnancy marks a useful point of contact, where women have regular medical appointments, allowing information and support to be provided. Our model is an excellent candidate for studying what impact a pregnancy treadmill exercise program may have on the future risk of cardiac disease development in the offspring. Whilst not all exercise and lifestyle intervention strategies during pregnancy have proved to have immediate measured benefit to the mother's lifestyle behaviour or clinical outcomes (see Section 1.10), our study demonstrates that it is still critical that follow up is undertaken to assess if exercise could reduce the long-term risks of CVD in the offspring. Even a small improvement in offspring risk could have important benefits for the future

burden of CVD worldwide (Norman and Reynolds, 2016). It is well documented that exercise intervention in an individual is sufficient to reduce their own risk of CVD (Adamu, Sani and Abdu, 2006). However, data from our study highlights that risk of CVD can be influenced as early as the developing fetus and therefore interventions to prevent CVD should start very early in life, and not only when the disease manifests later in life. The potential benefits of exercise intervention strategies during an obese pregnancy are not limited to effects on maternal health, but also improving the cardiovascular and metabolic health of the next generation.

4.4.8 Summary of key findings

Results of this chapter have shown:

- Maternal obesity caused pathological cardiac hypertrophy with increased interstitial fibrosis, this was prevented by maternal exercise intervention
- Male offspring of obese dams had accompanying hypertension and *in vivo* cardiac dysfunction.
- The observed hypertrophy was not a consequence of offspring hypertension, as the offspring of obese-exercised dams had hypertension without markers of cardiac hypertrophy.
- Despite the haemodynamic stress of elevated SBP, the offspring of obese-exercised dams displayed normalised parameters of cardiac function. This could have been achieved through a compensatory sympathetic-activated increase in inotropy (contractility).
- The discordance in the outcomes corrected by the exercise intervention suggests that the programming of cardiac dysfunction and hypertension occurs via divergent pathways.
- Maternal hyperinsulinemia is not required to programme offspring hypertension but could be involved in the mechanism of cardiac hypertrophy and dysfunction.
- Maternal hyperleptinemia that is not corrected by maternal exercise could instead programme offspring hypertension.

5. Cardiovascular phenotype in young adult female offspring

5.1 Introduction

5.1.1 Cardiovascular health of women

Traditionally, cardiovascular studies have been carried out in males. However striking gender differences exist in cardiovascular health which might mean findings established in males may not be applicable to females. CVDs are still the leading cause of death in women despite the dramatic decline in CVD mortality over the past three decades (Wilmot *et al.*, 2015). Recent data suggest stagnation in the improvements in incidence and mortality of CVD among younger women (<55 years) (Mozaffarian *et al.*, 2015). This finding demonstrates a need for a necessary change from studying CVD from a male only perspective. One study suggested that out of the patients presenting in hospital with myocardial infarction, women were more likely to present without chest pain and have a higher mortality then men in the same age group (Canto *et al.*, 2012). Presenting with atypical symptoms and the under-estimation of ischaemic heart disease risk can lead to a general under-diagnosis and under-treatment in women (Brewer, Svatikova and Mulvagh, 2015). Obesity tends to have a deeper impact on the risk of CVD in women. For example, the Framingham Heart Study showed that obesity increased the risk of CVD by 64% in women, compared to 46% in men (Sharma and Gulati, 2013). Hypertension prevalence is also higher in women (> 60 years old) then in men (Garcia *et al.*, 2016).

5.1.2 Cardioprotective effects in females

Some clinical studies have already revealed sex differences in CVD outcomes. Women that experience heart failure (HF) survive better than men and it also manifests at an older age and with less ischaemic aetiology (Levy *et al.*, 2002). Heart failure with preserved ejection fraction (HFpEF) is more common women than men (Duca *et al*.. 2018), this is one class of heart failure that has diastolic dysfunction and increased LV stiffness but with preserved left ventricular ejection fraction. Women with isolated systolic hypertension have increased wall thickness but with smaller chambers (concentric hypertrophy), whereas men are more likely to have LV dilatation without increased wall thickness (eccentric hypertrophy) (Krumholz, Larson and Levy, 1993).

Pre-menopausal females tend to have better cardiovascular outcomes then age-matched males (Luczak and Leinwand, 2009). After menopause this effect is largely lost and therefore the ovarian reserve hormone estrogen was suggested to be cardioprotective (Kim and Levin, 2006). Incidence of hypertrophy rapidly increases in post-menopausal women (Agabiti-Rosei and Muiesan, 2002) and this has been shown to be reversed by hormone replacement therapy (Miya *et al.*, 2003). This suggests estrogen could have anti-hypertrophic action and could prevent the upregulation of hypertrophic genes.

There are wide-ranging effects of estrogen on cardiac physiology, for example, the modulation of vascular tone, arterial resistance, arterial dilation, and blood flow. Also, effects include lowering BP, preventing the damage of arteries and the heart to various forms of injury, decreasing vascular inflammation, and atherosclerosis and preventing cardiac hypertrophy (Reviewed in: Kim and Levin, 2006). The mechanism by which estrogen mediates its cardioprotective effects is not fully known, however, it is known that estrogen activates NO production, alters Ca^{2+} flux and promotes cardiomyocyte survival through the PI3K-Akt pathway (Luczak and Leinwand, 2009).

5.1.3 Sex differences in developmental programming

As described in the previous section, there is a difference in the aetiology of cardiovascular disease between the sexes and this may be related to sex differences in myocardial function. Recent studies, however, have also demonstrated an intrinsic sex dichotomy in developmental programming through differential responses to adverse intrauterine environments. This could mean that male and female fetuses adapt differently to developmental stressors or that the sex hormones have an influence on the progression of developmentally programmed disease states.

Human studies have shown that there are sex differences in the way birthweight affects cardiovascular physiology; a smaller size at birth was associated with higher systemic vascular resistance in boys following stress whereas girls had increased sympathetic activation both at rest and during stress (Jones *et al.*, 2008). Autonomic cardiovascular control was altered between the sexes such that women, but not men, who were small at birth demonstrated increased BP variability and reduced baroreflex sensitivity (Jones *et al.*, 2007).

The Dutch Hunger Winter famine study (Section 1.4.1) showed offspring with phenotypes that depended on both sex and timing of exposure. Only females demonstrated increased BMI,

waist circumference and adiposity when exposed to famine *in utero,* and this was later shown to be accompanied by a disrupted lipid profile (Ravelli *et al.*, 1999; Lumey *et al.*, 2009). Developmental programming by a diabetic mother has a stronger impact on glucose homeostasis and BP in the female offspring, suggesting an increased sensitivity to changes in glucose availability during early life, when compared to males (Krishnaveni *et al.*, 2010). The effect of maternal pre-pregnancy BMI on offspring weight, height and BMI growth patterns from 0 to 7 years of age was stronger in females than males (Oostvogels *et al.*, 2017). Sex-specific responses to developmental programming stimuli have also been reported in a wide range of animal models including rodent and large animal models (reviewed in: Aiken & Ozanne 2013). These studies highlight the importance of including both sexes in programming studies as there is a need to understand how changes in the early life environment impact differently on long-term health outcomes of male and female individuals.

Male and females undergo development at different rates, *in utero* the male grows faster and therefore experience a greater exposure to the insult, and this has been suggested for one of the reasons that males appear to be more deeply impacted (Aiken and Ozanne, 2013). There are several mechanisms mediating sexual dimorphic programming, including epigenetics, sex differences in the anatomy and growth of the placenta, the protective effects of estrogen, and finally the difference in the circulating levels and sensitivity to metabolic hormones (Dearden, Bouret and Ozanne, 2018).

5.1.4 Aims of chapter

The first aim of this chapter was to investigate the effect of maternal obesity on the female offspring's cardiovascular health. Once this was established, the second aim was to determine the impact on cardiovascular function of the maternal exercise intervention. Comparing the results with Chapter 4, will highlight sex differences in the effects of maternal obesity on the cardiovascular health of the offspring.

5.1.5 Disclaimer

The detection of mites in the animal facility meant all experiments and breeding had to be halted for six weeks to allow for effective treatment. The chosen treatment was permethrin based and concerns were raised about its impact on breeding and feeding behaviours (Bloom, Staatz and Dieringer, 1983; Zhang *et al.*, 2007). This had the unfortunate consequence that no animal experiments could take place between July 2017 and January 2018; this included the treatment and quarantine period plus the time for the offspring to be generated again using our

diet-induced obesity model. The timing of this resulted in the data for the intervention group being incomplete. Therefore, although sufficient numbers of animals for all physiological measurements were successfully generated and experiments carried out, this down time meant there was not sufficient time to generate enough animals to carry out molecular and stereological analyses. Therefore this analysis was performed only in the control and obese offspring groups using existing banked fixed and frozen tissue. Hearts from the physiological experiments could not be used for molecular analysis due to the exposure to isoflurane after echocardiographic study.

5.2 Methods

5.2.1 Cardiomyocyte cell size analysis: WGA and HALO™

This method has been further optimized since it was carried out in Chapter 4, as the department purchased new equipment and software for this type of analysis. This enabled imaging of the whole heart and not just the LV. Perfusion-fixed hearts from eight week old offspring were processed and sectioned at 3 µm. Two mid-cardiac sections from each heart were selected for staining using a method as previously described (Blackmore *et al.*, 2014). Six hearts (from six independent litters) were analysed per experimental group. Wheat germ agglutinin [TexasRed-X conjugate, (Molecular Probes, Life Technologies, USA)] was used to stain cardiomyocyte cell borders. The staining protocol is the same as has been described in section 4.2.2. The slides were imaged using an automated method with the Slide Scanner Axio Scan Z1 (Zeiss, Germany). Effort was taken to ensure a high quality of staining and imaging to allow for accurate analysis, therefore when this was considered suboptimal, the staining was repeated.

Images were analysed using the muscle fiber module of the HALO™ analysis software (Indica labs). An algorithm was used to detect cell borders and automatically circumscribe the cells. Minimum and maximum fiber area, a measure of roundness and maximum segmentation length were fixed to ensure the cell areas were measured similarly. The ventricle was manually annotated and then the analysis was performed within the annotated area (Figure 5.1). This enabled comprehensive analysis of all cells in each ventricle that lie in cross section, unlike analysis performed in section 4.2.2 where 10 random images were taken of the LV. Two mid-cardiac sections were analysed per animal.

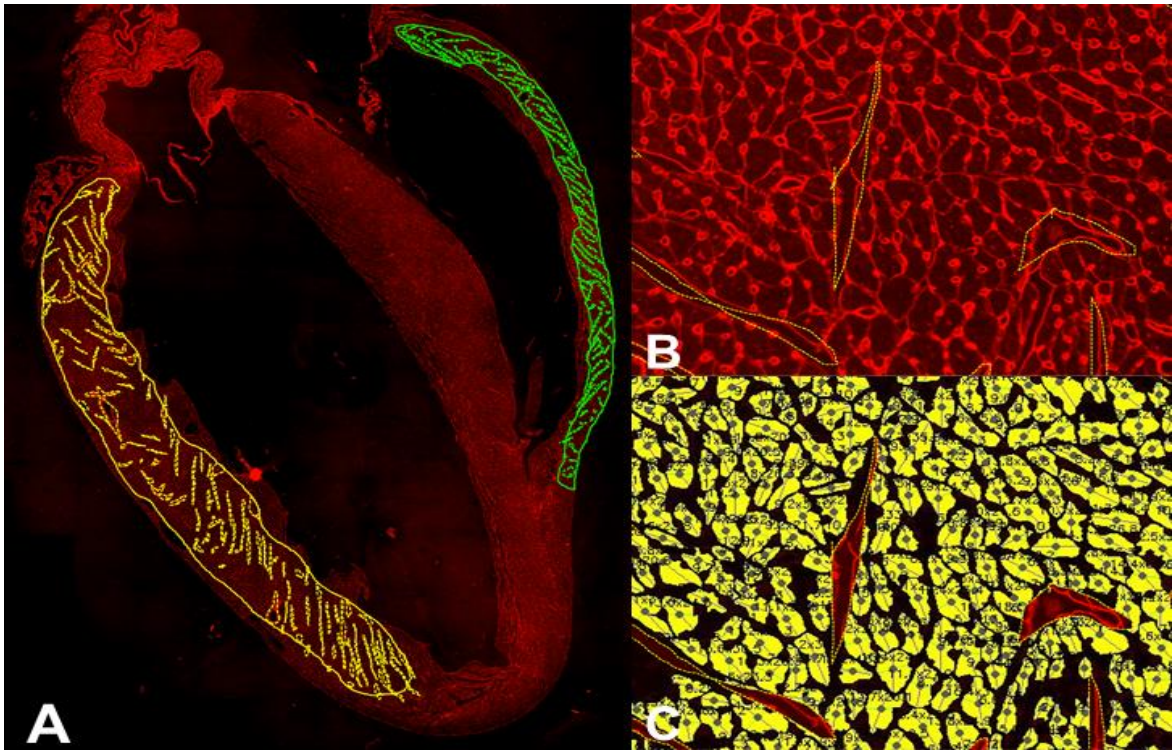


FIGURE 5.1: Representative images of HALO™ WGA analysis. A) Annotations drawn around ventricles in separate analysis layers. B) Image showing WGA-stained cardiomyocytes with areas that are excluded from analysis (yellow dotted line) C) HALO™ analysis of circumscribing cell borders.

5.2.2 Ventricular and lumen dimensions in mid-cardiac sections

Images of the mid-cardiac section that were stained and imaged for cell size/area analysis (see section 5.2.1) were analysed for ventricular widths and areas. The experimental group was blinded to the analyser. A previously published stereological method was utilised (Blackmore *et al.*, 2014). Images were exported as TIFF format ensuring that a scale bar was added. Images were then analysed using FIJI software. Horizontal lines with equal spacing was superimposed onto the image to enable the unbiased assessment of ventricle wall widths. Where the superimposed line intersected with the edge of the ventricle wall a line to measure the width was drawn. An average (mean) was used of least 10 measurements taken down the length of the both ventricle walls (Figure 5.2A).

Ventricle area was assessed by superimposing crosses onto the image where the area of the cross was known. The plugin (available on FIJI) called Cell counter was used to count the number of crosses falling on the LV, left lumen (LL), right ventricle (RV), right lumen (RL) and IVS (Figure 5.2B). This was analysed over two mid-cardiac sections and the average was calculated.

The Cavalieri principle was then used to calculate area of ventricles:

$Area\ (mm^2) = A(p) \times \sum p$, *A(p) = known area/point.* Σp = *Sum of each point counted.*

Total LV area was considered to be the sum of the LV and the IVS area.

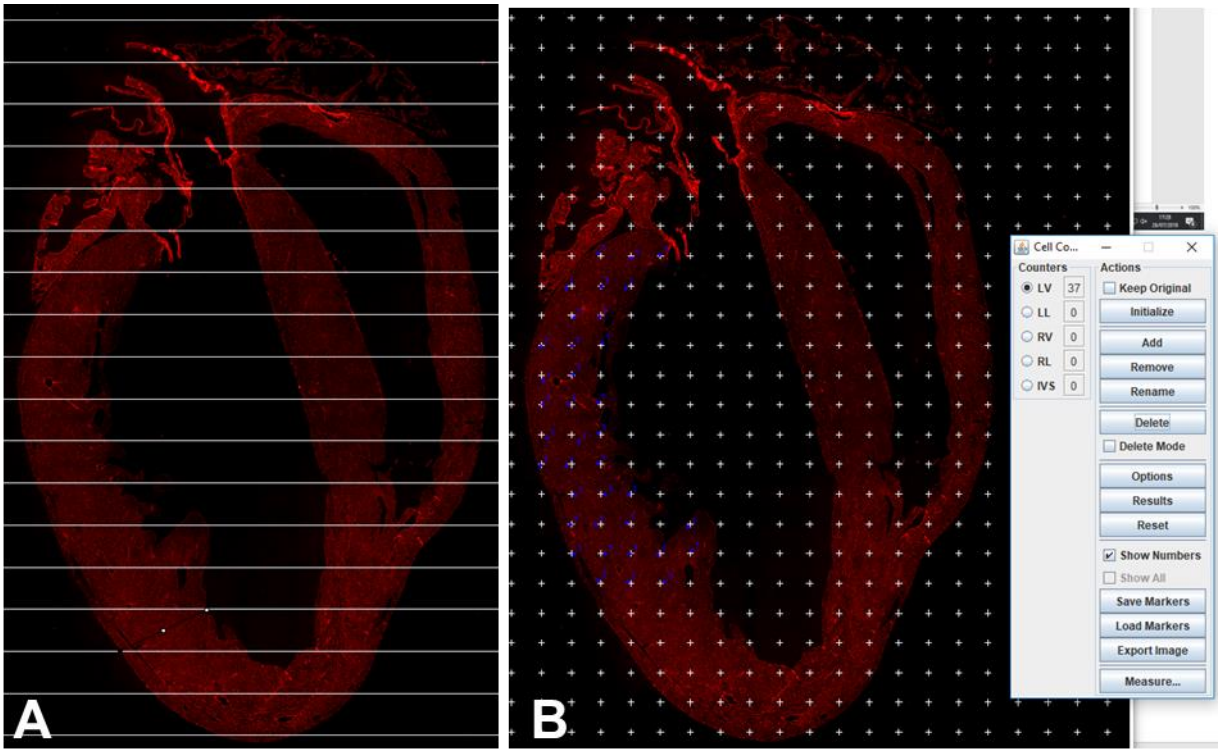


FIGURE 5.2: Representative images from FIJI analysis. This highlights the method used to determine ventricle width and area measurements. A) Wall width was measured where superimposed line intersects edge of wall to the neighbouring superimposed line. B) Ventricle areas were analysed by counting number of crosses that fall on the ventricles, lumens and IVS.

5.2.3 PCR: primer sequences

Gene expression was measured in ventricular heart tissue. The PCR protocol including how primers were designed is described in Section 2.6. The primer sequences used in this Chapter are shown in Table 5.1.

TABLE 5.1: Primer sequences for PCR. A geomean of 3 housekeepers (in **bold**) was used to normalize the gene expression data in this chapter, *Tbp*- TATA-binding protein, *Rpl4*- Ribosomal protein L4 and *Eef1e1*- Eukaryotic translation elongation factor 1 epsilon 1.

	Forward primer (5'-3')	Reverse primer (5'-3')
Tbp	TATGACCCCTATCACTCCTG	TTCTTCACTCTTGGCTCCTGT
Rpl4	GCCGCTGGTGGTTGAAGATAA	CGTCGGTTTCTCATTTTGCCC
Eef1e1	TCCAGTAAAGAAGACACCCAGA	GACAAAACCAGCGAGACACA
Fetal genes see Section 4.2.3		
Antioxidant defence genes see Section 4.2.3		
SERCA2	CTCCATCTGCTTGTCCAT	GCGGTTACTCCAGTATTG

5.2.4 PicroSirius red stain: Fibrosis analysis

The full protocol is described in Section 4.2.4.

5.2.5 Non-invasive blood pressure measurement

Full detailed methodology can be found in Section 2.4.

5.2.6 Echocardiography: Aortic width analysis

Full methodology is described in Section 2.5

5.2.7 Western blotting

The method for western blot is described in Section 2.8 and details of antibodies (including dilutions) are the same as those shown in Section 4.2.7. Coomassie stained gel to ensure even protein loading shown in Figure 5.3.

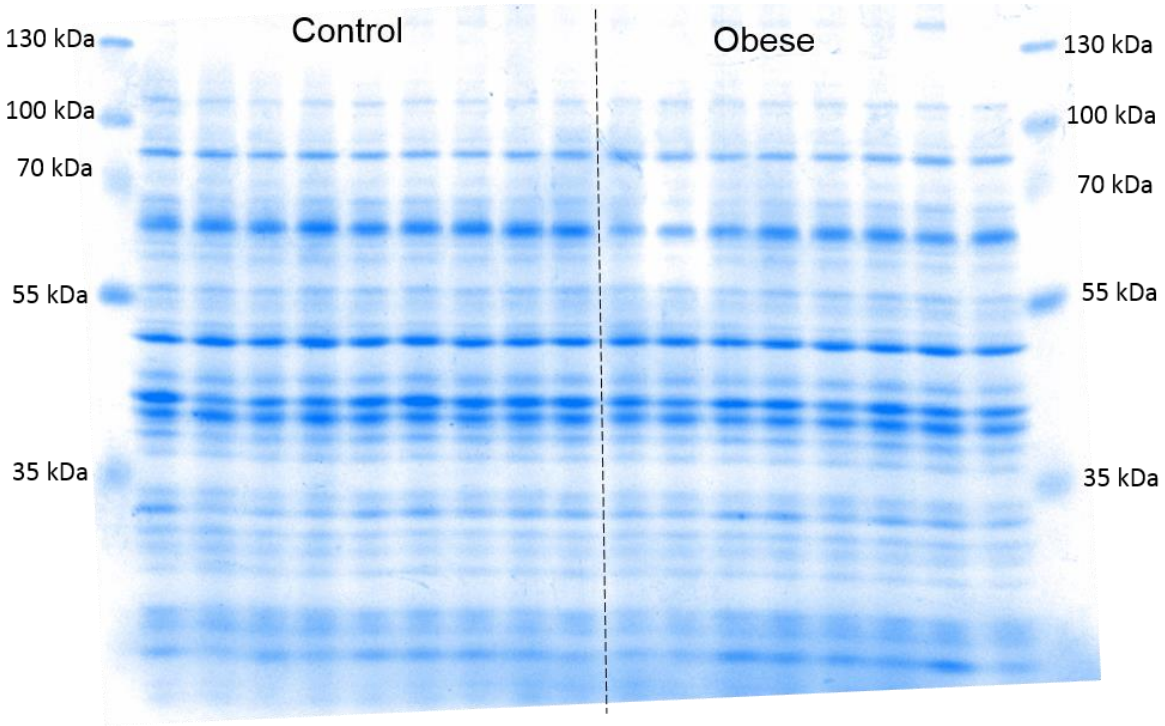


FIGURE 5.3: Coomassie stain of loaded gel to ensure even protein loading. Control *n*= 9 and Obese *n*= 8.

5.2.8 Statistical analysis

When analysis was performed between two groups (offspring of control dams vs. offspring of obese dams) an unpaired parametric t-test was utilised. One-way ANOVA with Bonferroni post-hoc test was performed when three groups were compared. TD-NMR measurements were analysed by two-way ANOVA with Bonferroni post-hoc test. The hierarchical linear model used to assess cardiomyocyte cell area was described in Section 4.2.8; the ventricles were considered in separate models. Cardiomyocyte area frequency distributions were plotted in R statistical software package version 2.14.1 (R Foundation for Statistical Computing, Austria) due to large number of data values. All data is presented as mean ± SEM.

5.3 Results

5.3.1 Body composition and organ weights

Longitudinal assessment of bodyweight and body composition were carried out, and a two-way ANOVA used to assess statistical significance as due to missing data a correction for repeated measures could not be carried out. Age had a significant effect on bodyweight, fat mass and lean mass, which all increased with age (Figure 5.4A-C). There was no significant

effect of maternal diet on bodyweight in the female offspring (Figure 5.4A). There was a significant effect of maternal diet on female offspring fat mass, with the ANOVA analysis suggesting that maternal obesity led to increased adiposity (Figure 5.4B). Lastly there was an effect of maternal diet on lean mass with female offspring of obesogenic diet-fed dams showing decreased lean mass compared to control (Figure 5.4C).

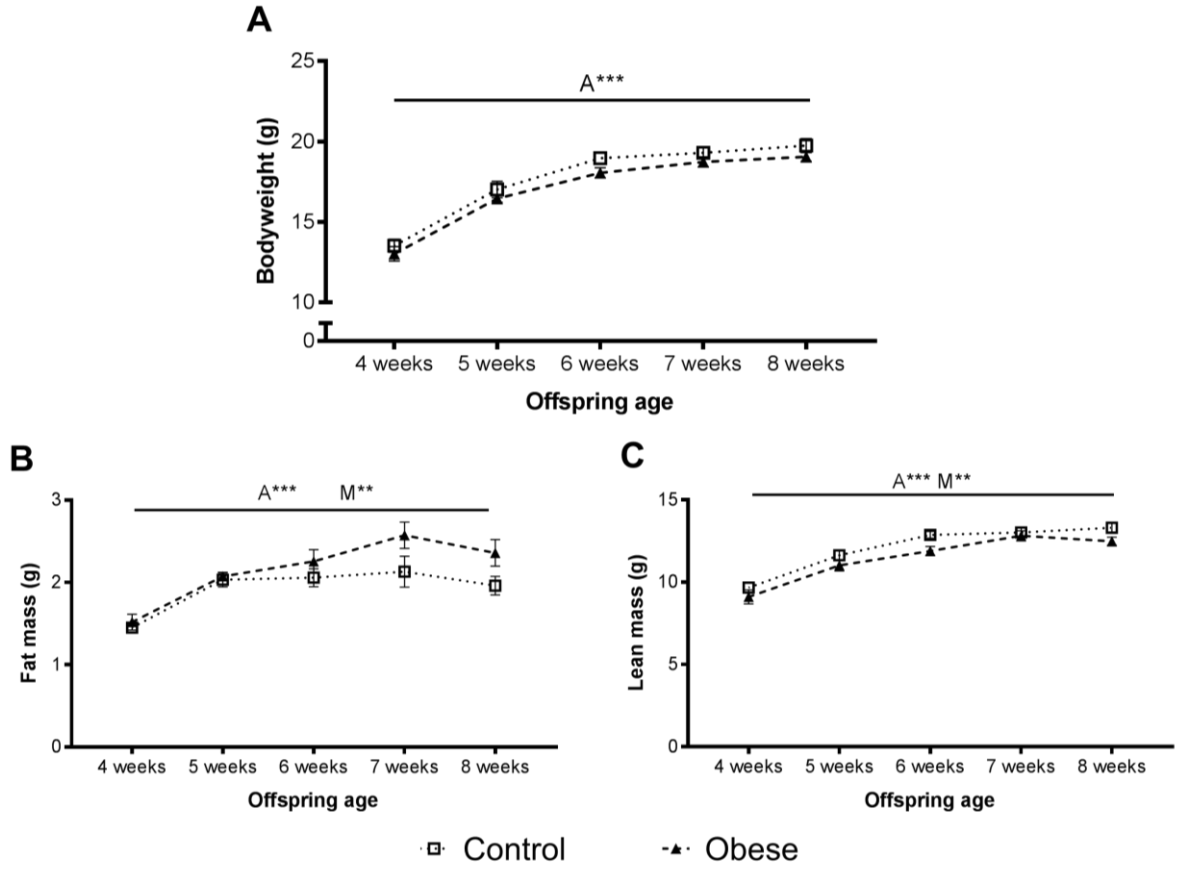


FIGURE 5.4: Longitudinal assessment of body composition. Body composition measured by TD-NMR from four weeks of age to eight weeks of age in female offspring. TD-NMR measurements were carried out at the same time of the day each week (Morning) to account for different feeding behaviours in light/dark cycle. Control *n*= 8 and Obese *n*= 8. Two way ANOVA; A*** effect of age $p< 0.001$, M** effect of maternal diet $p< 0.01$.

Body composition assessed at eight weeks of age was plotted individually and there was no difference in bodyweight, fat mass or lean mass between groups (Table 5.2). Female offspring were fasted for 16 hours (overnight) prior to *post mortem* at eight weeks of age. There were no differences in the fasted bodyweight or in the bodyweight lost during the fast (Table 5.2). There were no differences in fat depot weights in the offspring of obese dams. There were also no differences in other organ weights measured in Table 5.2 between offspring of control and obese dams.

TABLE 5.2: Body composition and organ weights in eight week old female offspring. TD-NMR measured body composition in the fed state. Data is presented mean ± SEM. Females were fasted for 16 hour before post mortem where organs were dissected and the wet tissue weight was recorded. *P* values calculated from unpaired t-test.

Body composition + organ weights (g)	*Control*	*n*	*Obese*	*n*	*p value*
Fed bodyweight	19.76 ± 0.43	8	19.06 ± 0.27	8	0.196
Fat mass	1.96 ± 0.11	8	2.20 ± 0.15	8	0.228
Lean mass	12.85 ± 0.38	8	11.74 ± 0.61	8	0.150
Fasted bodyweight	17.97 ± 0.49	6	17.22 ± 0.53	6	0.320
Bodyweight lost with fast	2.17 ± 0.22	6	1.75 ± 0.17	6	0.167
Gonadal fat	0.165 ± 0.023	6	0.191 ± 0.027	6	0.491
Retroperitoneal fat	0.041 ± 0.009	6	0.033 ± 0.008	6	0.589
Liver	0.86 ± 0.03	6	0.85 ± 0.04	6	0.949
Kidney; left	0.121 0.002	6	0.120 ± 0.005	6	0.784
Kidney; right	0.110 ± 0.003	6	0.113 ± 0.005	6	0.631
Pancreas	0.098 ± 0.006	6	0.104 ± 0.008	6	0.584
Spleen	0.064 ± 0.004	6	0.06 ± 0.004	6	0.465

5.3.2 Serological analysis

There were no significant differences in fasting plasma glucose from tail blood before the *post mortem* (Figure 5.5A). Female offspring of obese dams showed increased free fatty acids (FFAs) compared to control (Figure 5.5C) but there were no further differences in the measured metabolites (Figure 5.5B+D). There were also no differences between groups in serum leptin or insulin when ELISA was performed (Figure 5.5E+F).

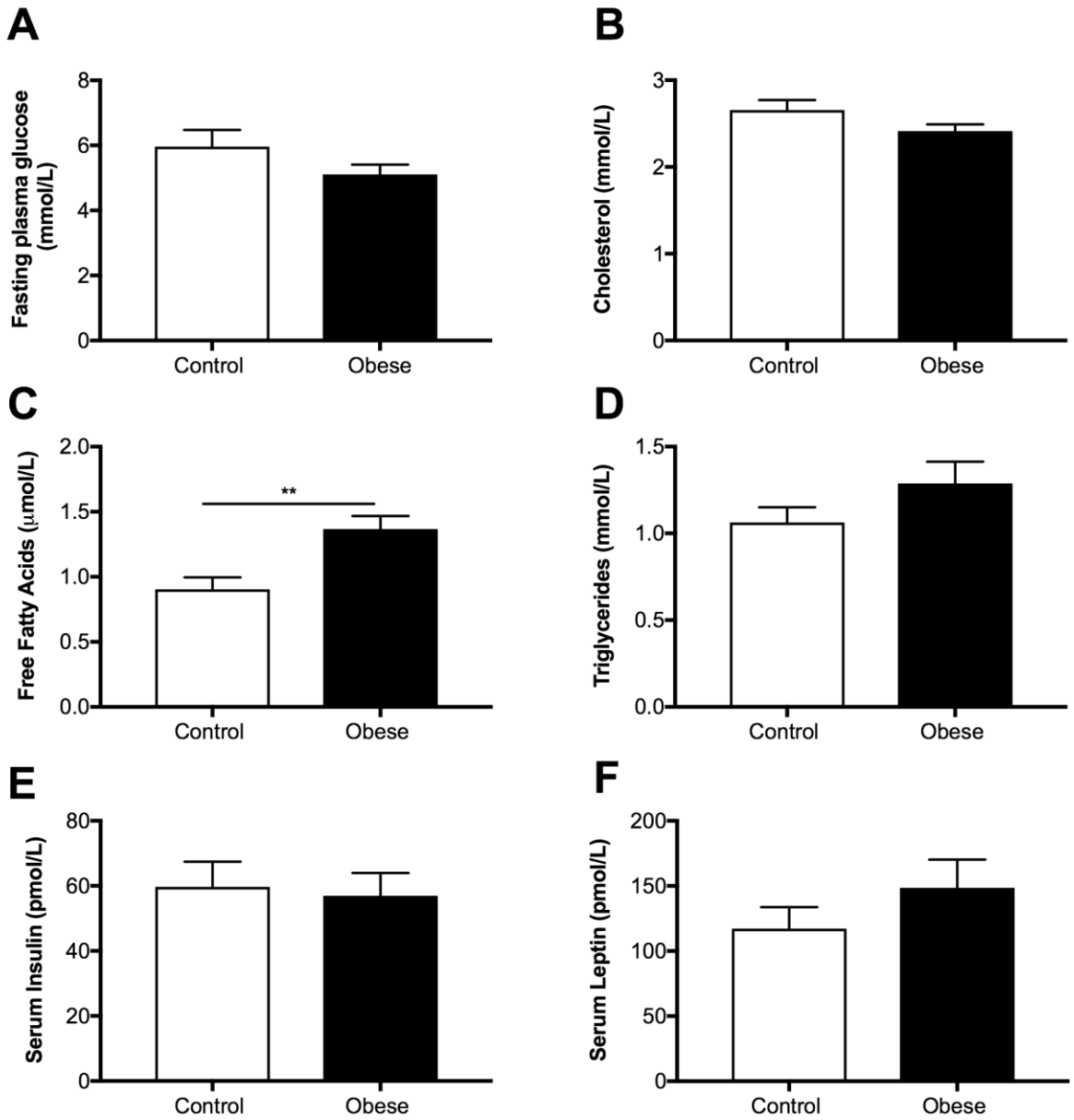


FIGURE 5.5: Serological analysis in 16 hour fasted serum. Methods for serum analysis is described in Section 2.3. Control *n*= 8 and Obese *n*= 8. Except for cholesterol (***2 outliers*** were detected by Grubbs test) Control ***n= 7*** and Obese ***n= 7***. FFAs (***1 outlier*** detected) Control ***n= 7*** and Obese *n*= 8. Unpaired t-test; ** $p< 0.01$.

5.3.3 Cardiac phenotype

Increased heart weight

At eight weeks of age, female offspring of obese dams had increased heart weight, when expressed as absolute weight (Figure 5.6A) and when expressed relative to bodyweight (Figure 5.6B). Heart weight was normalized to the fasting bodyweight measured immediately before *post mortem* and the fasted bodyweight was not different between groups (Table 5.2).

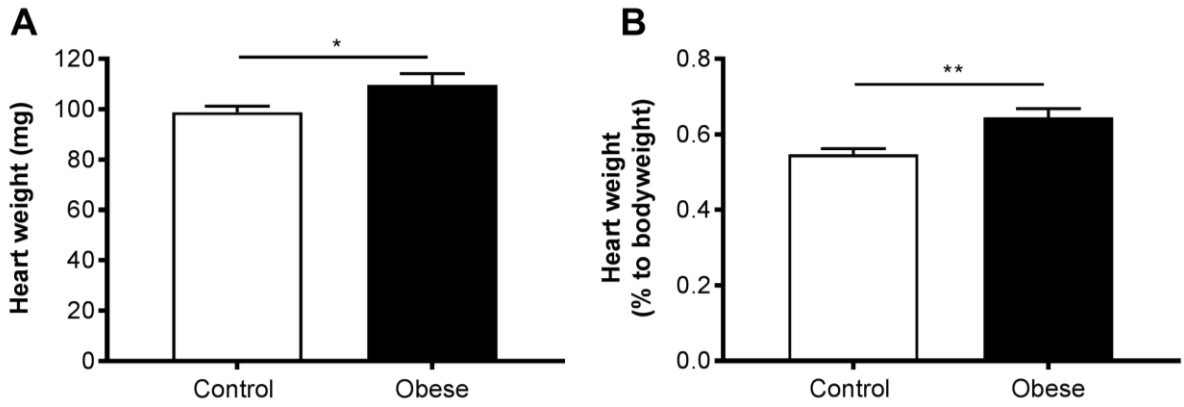


FIGURE 5.6: Heart weights at *post mortem*. Heart weight of female offspring was measured by weighing wet tissue weight at *post mortem* post 16 hour fast. Heart weight was also expressed relative to bodyweight. Control n= 8 and Obese n= 7. Unpaired t-test; * p< 0.05 and ** p< 0.01.

Right ventricular hypertrophy

Cardiomyocyte cell area was assessed in mid-cardiac sections of perfusion fixed hearts. Both ventricles were assessed and increased mean cardiomyocyte cell size was observed in the RV, but not the LV, of the offspring of obese dams (Figure 5.7A+B). The frequency distribution of cardiomyocyte cell area has a rightward shift suggesting female offspring of obese dams had more cells of larger area in their RV (Figure 5.7D). A hierarchical linear model was employed to stringently assess the cardiomyocyte cell area of each ventricle separately. For the LV there was no significant effect of maternal diet on cell size (p= 0.13) but for the RV, there was a significant effect (p< 0.05).

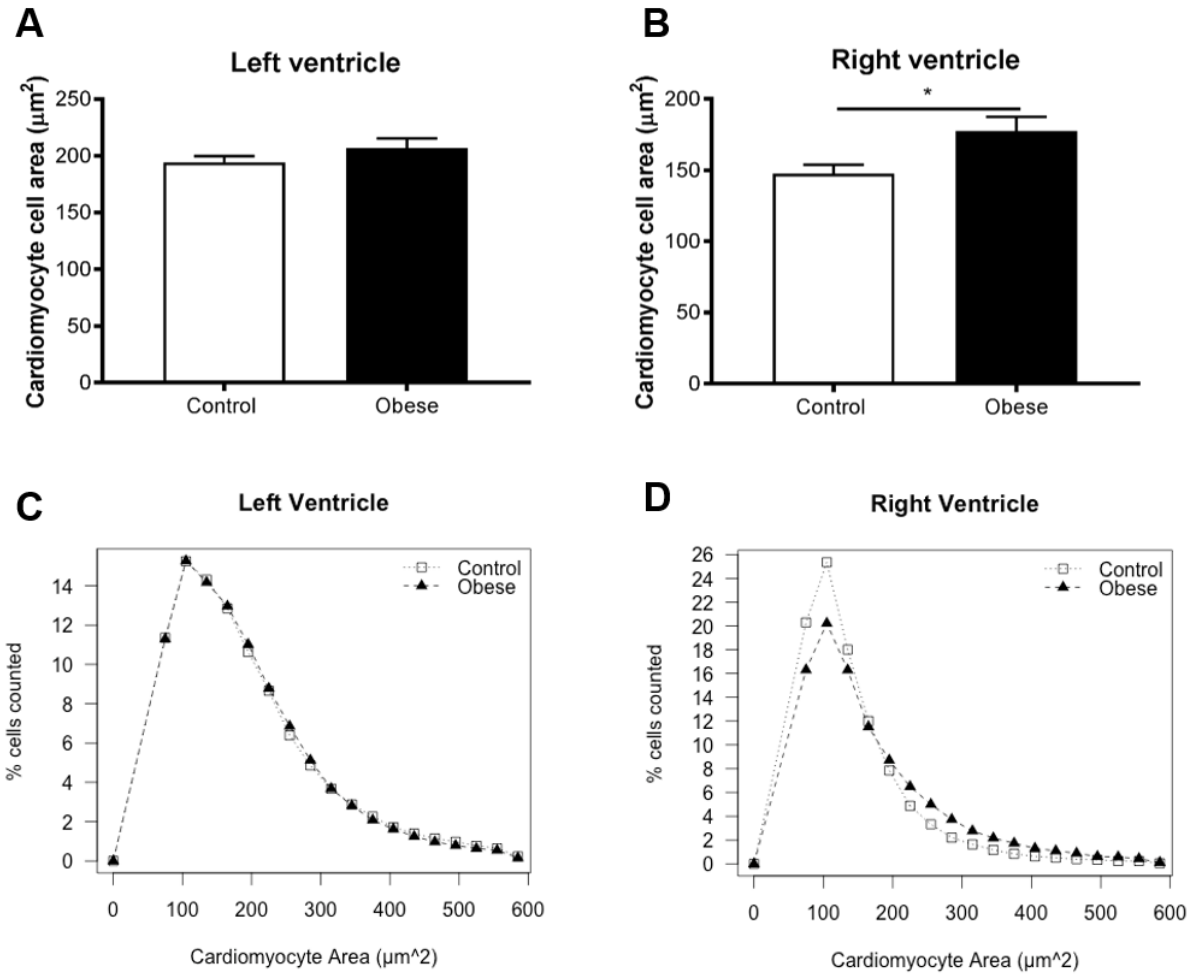


FIGURE 5.7: Cardiomyocyte cell size analysis. Average cardiomyocyte cell area across A) LV and B) RV. Two mid-cardiac sections analysed per heart and *n*= 6 hearts analysed per group. One-way ANOVA with Bonferroni post-hoc test; * $p< 0.05$. Frequency distribution of cardiomyocyte cell area in C) LV and D) RV.

Full comprehensive stereological analysis across the whole heart was not possible (due to time constraints). To complement and support the cell area data, analyses of ventricular wall and lumen dimensions was therefore performed on the same mid-cardiac sections as the cell area analysis. The wall thickness/width of the LV was not altered in the female offspring by maternal obesity and the wall: lumen ratio was similarly unaltered (Figure 5.8A+B). A significant increase in RV wall width in the female offspring of obese dams was seen (Figure 5.8C). There was no statistical difference in the RV wall: lumen ratio (Figure 5.8D). The LV: RV wall width ratio was decreased as a result of the increase in RV wall width (Figure 5.8E).

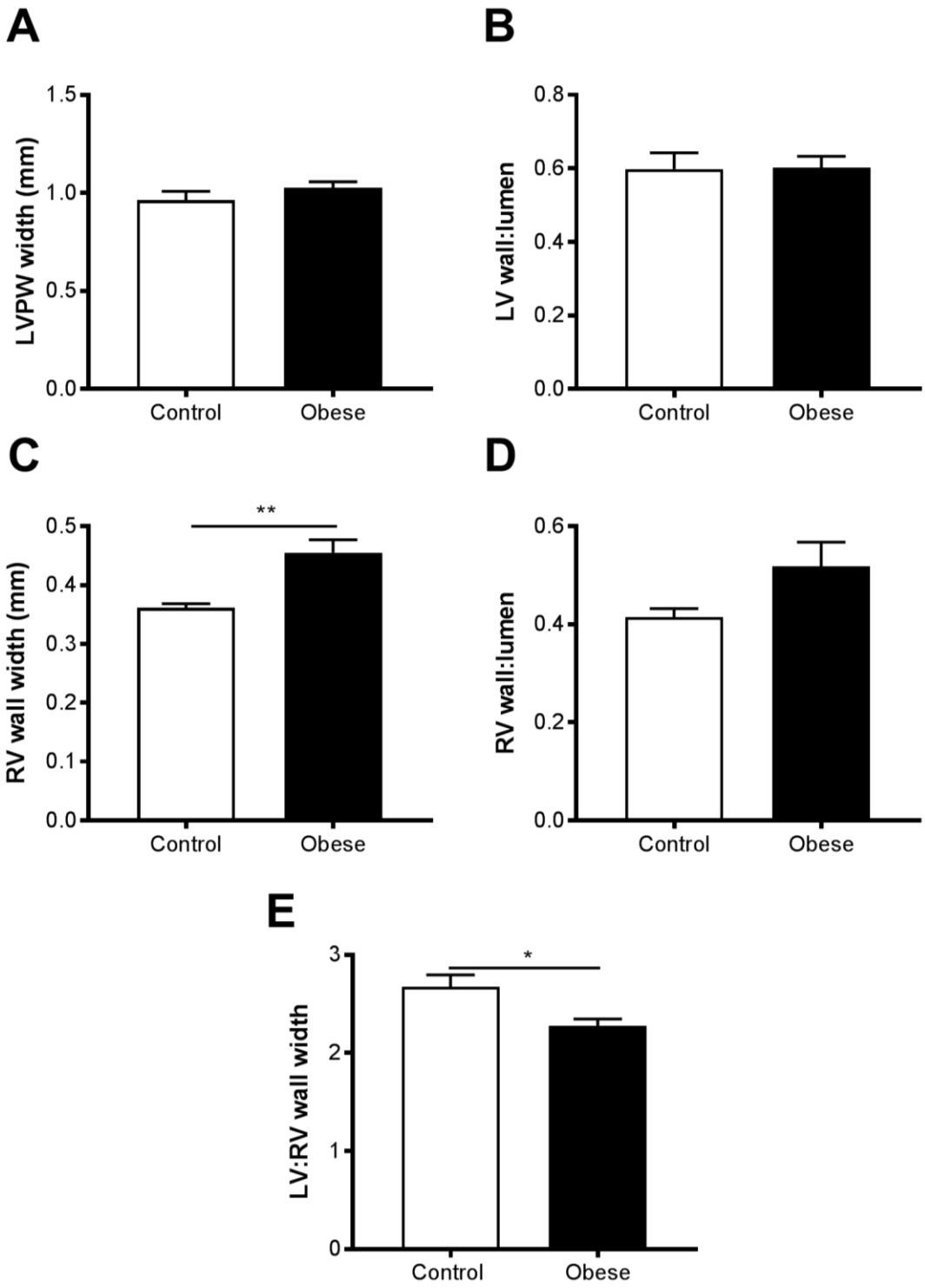


FIGURE 5.8: Mid-cardiac ventricle wall width analysis. Two mid-cardiac sections were analysed for ventricular wall widths. A) LVPW, B) LV wall: lumen widths, C) RV wall width, D) RV wall: lumen widths and E) LV: RV width ratio. Control *n*= 6 and Obese *n*= 6. Unpaired t-test; * $p< 0.05$ and ** $p< 0.01$.

LV area was not altered in the hearts of the female offspring after exposure to maternal obesity (Figure 5.9A+B). RV area was increased in the female offspring of obese dams (Figure 5.9C), and the RV area relative to total heart weight was also increased (Figure 5.9D).

This caused a reduction in the LV: RV area ratio (Figure 5.9E). Total heart area of the mid-cardiac section was also increased in female offspring of obese dams (Figure 5.9F).

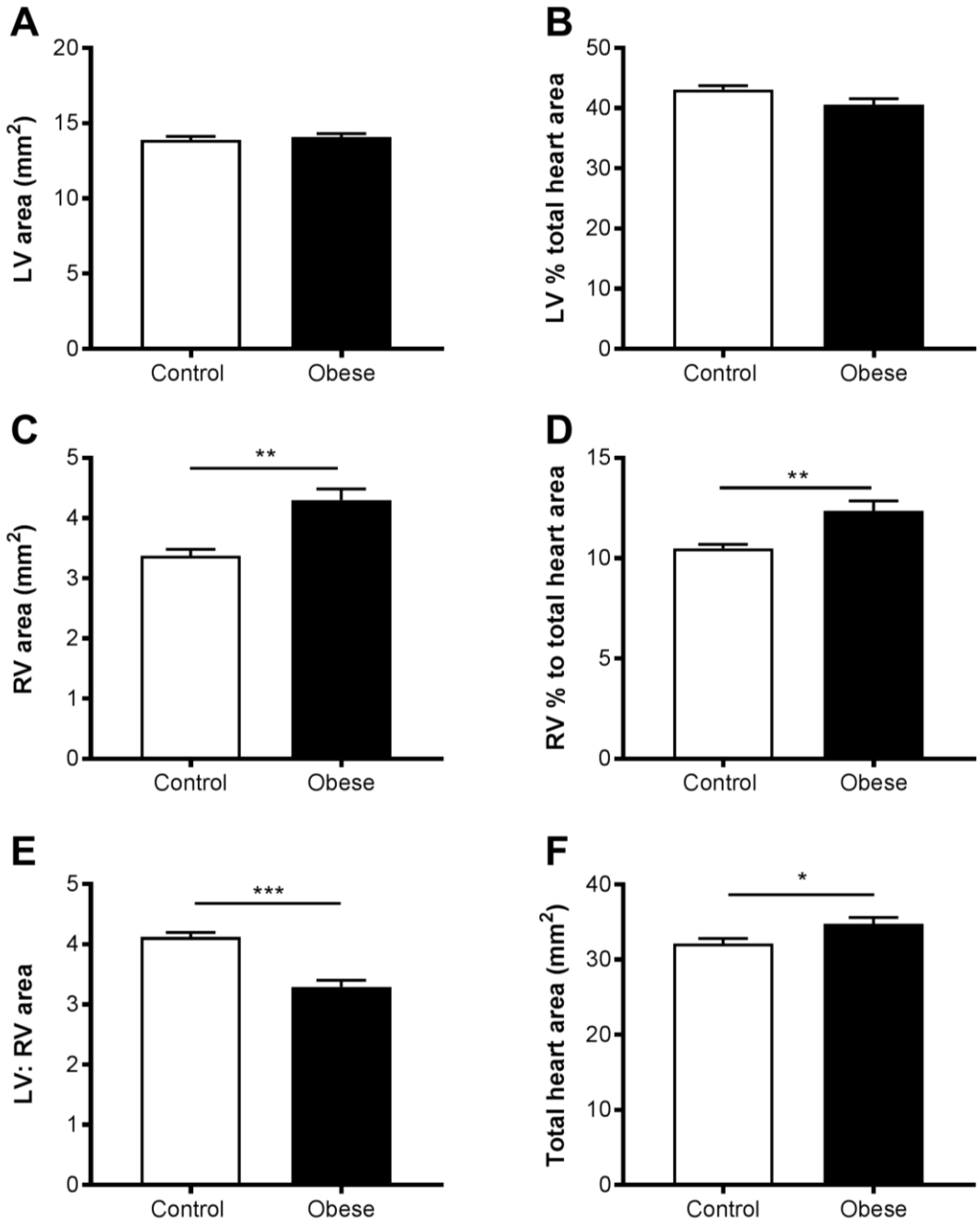


FIGURE 5.9: Mid-cardiac ventricular and total heart area analysis. A) LV area, B) LV area relative to total heart area (%), C) RV area, D) RV area relative to total heart area (%), E) LV: RV area ratio and F) Total heart area of mid-cardiac section. Control *n*= 6 and Obese *n*= 6. Unpaired t-test; * $p< 0.05$ and ** $p< 0.01$.

Alongside the heart weight, cell size and mid-cardiac data, the mRNA expression of pathological cardiac hypertrophy markers was measured in ventricular tissues. The expression

of *Nppa* and *Myh7* were increased compared to offspring of control dams (Figure 5.10A+E). The expression of *Myh6* was unaltered between groups (Figure 5.10D), however the increased expression of *Myh7* in the offspring of obese dams did not significantly alter the *Myh7:Myh6* ratio (Figure 5.10F).

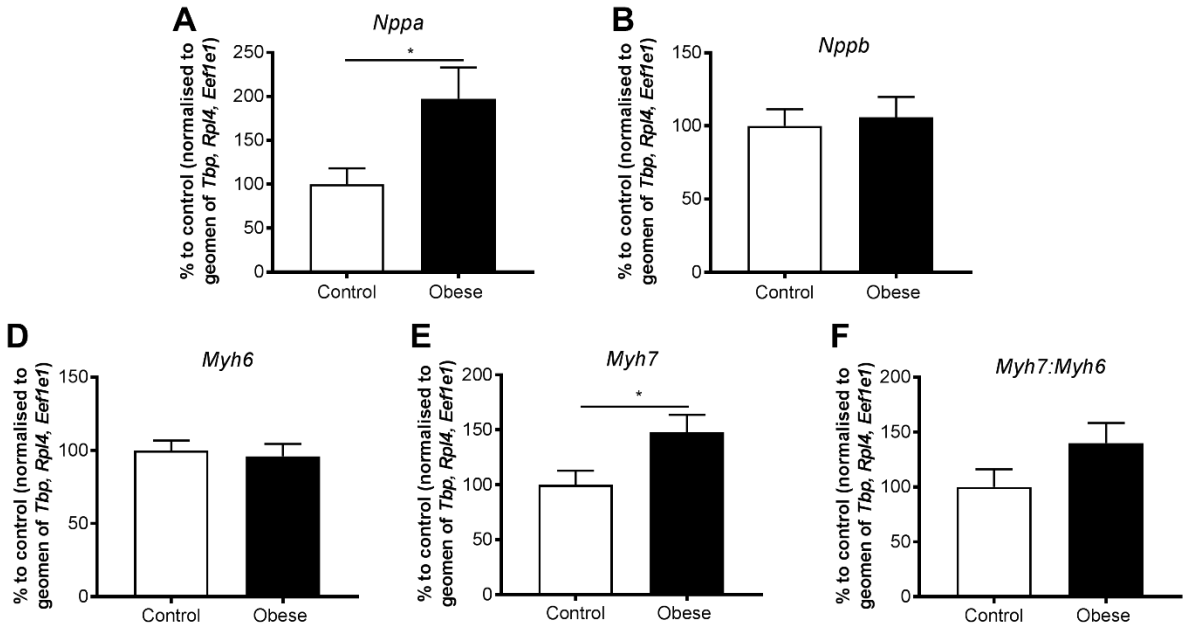


FIGURE 5.10: Markers of pathological cardiac hypertrophy. Gene expression of key fetal genes in the ventricular tissue of female offspring. Normalised to the geometric mean of three housekeepers: *TBP*- TATA-binding protein, *Rpl4*- Ribosomal protein L4 and *Eef1e1*- Eukaryotic translation elongation factor 1 epsilon 1. Control *n*= 7 and Obese *n*= 7. Unpaired t-test; * $p< 0.05$.

Cardiac Fibrosis

Mid-cardiac sections were stained with PicroSirius red to allow for the assessment of cardiac fibrosis (method described in Section 4.2.4). Representative images are shown in Figure 5.11A and analysis showed that there were no differences in % area of fibrosis between groups (Figure 5.11C). There were also no differences in the mRNA expression of antioxidant defence genes (Figure 5.11D).

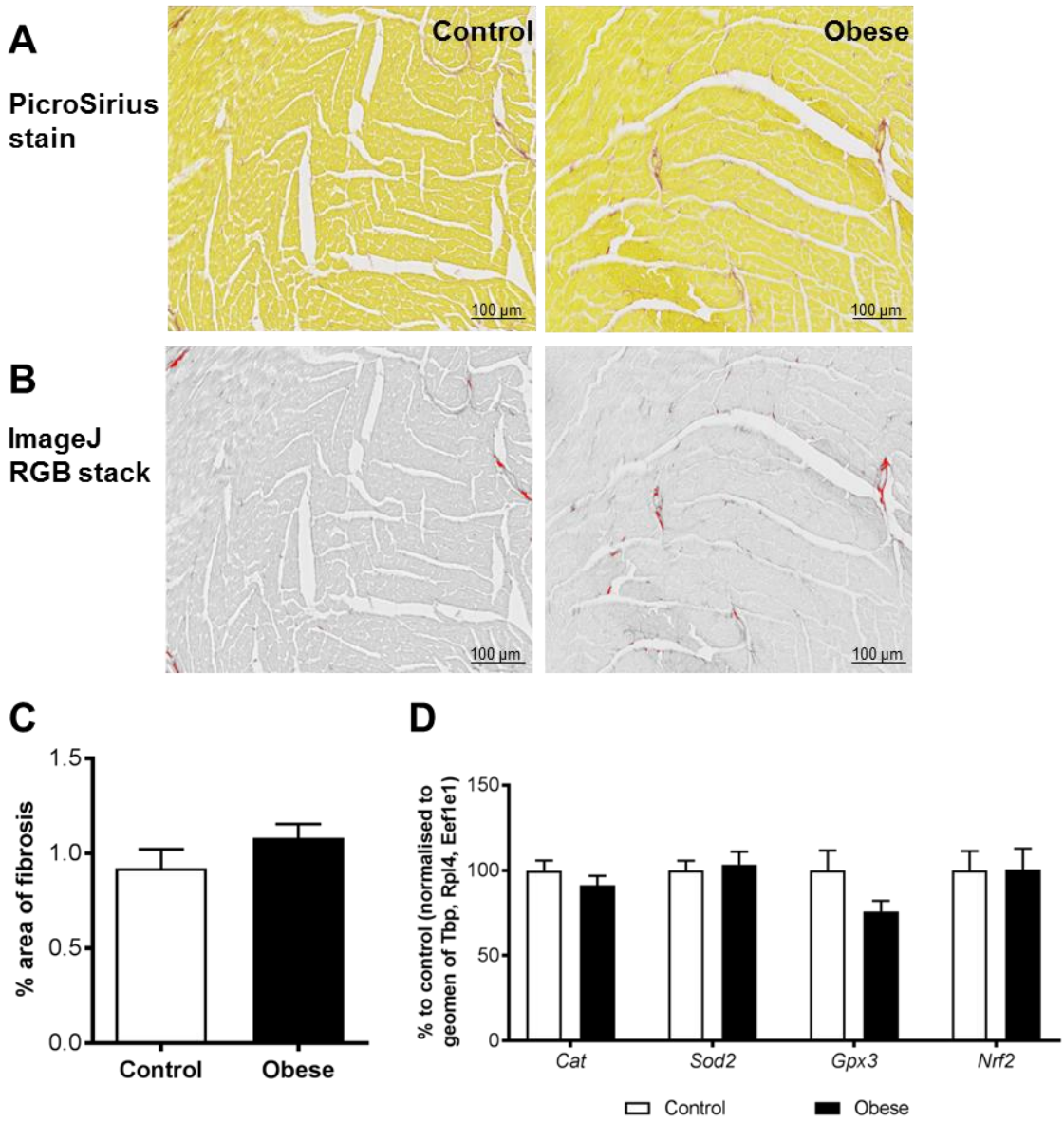


FIGURE 5.11: Assessment of cardiac fibrosis and oxidative stress. Method is detailed in Section 4.2.4. A) Representative images of PicroSirius red stained heart sections. B) Images were converted into RGB stack in ImageJ to allow for analysis. C) % area of fibrosis (% area of red stain) was assessed in three groups. Control *n*= 4 and Obese *n*= 4. Heart sections for staining provided by Dr Sarah Carr. D) Expression of antioxidant defence genes: *Cat*- Catalase, *Sod2*- Superoxide dismutase 2, *Gpx3*- Glutathione peroxidase and *Nrf2*- Nuclear factor erythroid 2–related factor 2. Expression normalized by geometric mean of *Tbp*- TATA-binding protein, *Rpl4*- Ribosomal protein L4 and *Eef1e1*- Eukaryotic translation elongation factor 1 epsilon 1. Control *n*= 8 and Obese *n*= 7.

Contractile machinery protein expression

The expression of key contractile machinery proteins was assessed and showed no significant differences in any of them between the female offspring of obese and control dams. However, the expression of phosphorylated troponin I and the phos:total troponin I ratio appeared reduced in the offspring of obese dams (Figure 5.12B+C). The protein expression of SERCA2 was not statistically different (Figure 5.12E) however when this was assessed at the mRNA level this was significantly reduced in the offspring of obese dams (Figure 5.12F).

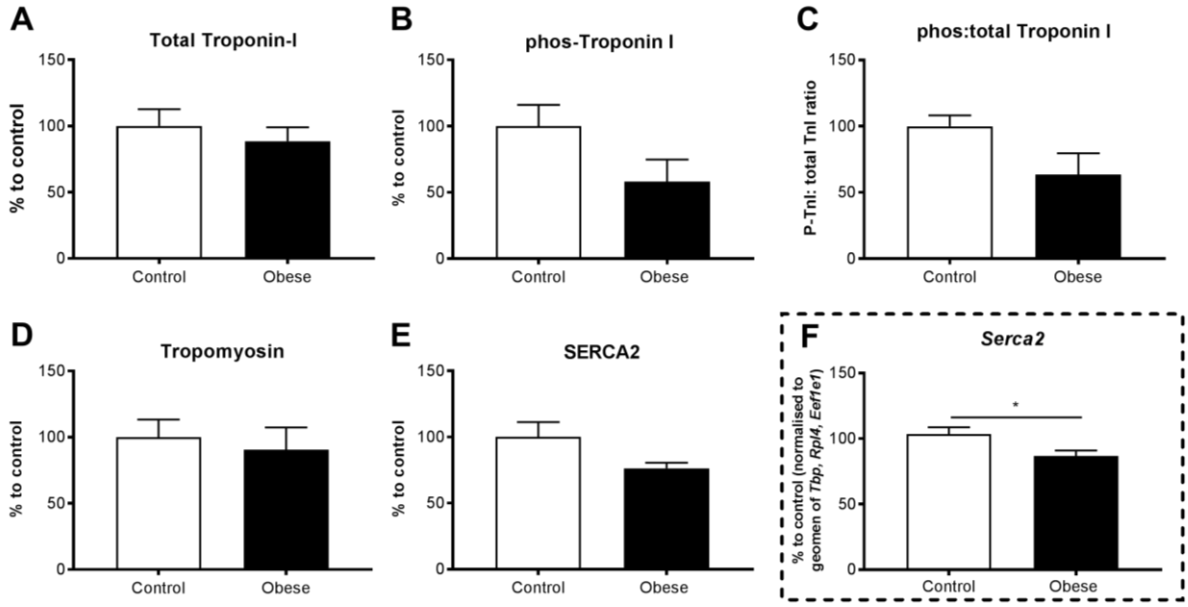


FIGURE 5.12: Expression of key contractile machinery proteins. A)-E) Expression level assessed by western blotting. F) mRNA expression by PCR of *Serca2* gene. Control *n*= 8 and Obese *n*= 7. Unpaired t-test; * $p< 0.05$.

Offspring blood pressure

Female offspring of obese dams showed elevated SBP and this was not prevented by the exercise intervention (Figure 5.13A). The increased SBP was not accompanied by any changes in pulse rate (Figure 5.13B).

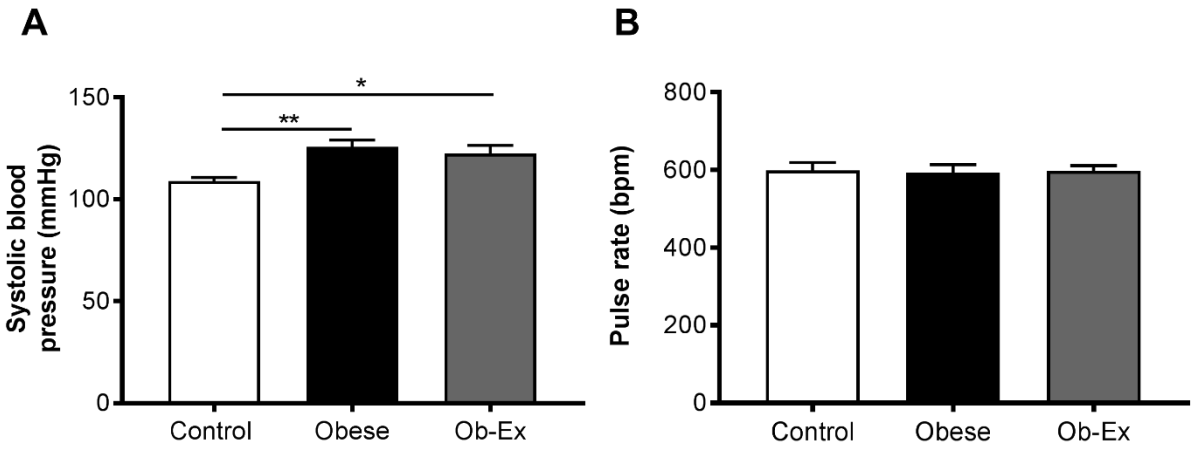


FIGURE 5.13: Female offspring SBP and pulse rate. A) BP measurements were carried out just prior to the start of the dark (active) cycle, at the same time (1600) across three consecutive days. Measurements were taken on the 3rd day when variation between measurements was lowest. This is fully described in Section 2.4. B) Pulse rate (bpm). Control *n*= 16, Obese *n*= 14 and Ob-Ex *n*= 10. One-way ANOVA with Bonferroni post-hoc test; * $p< 0.05$ and ** $p< 0.01$.

In vivo cardiac function

Unlike the male offspring (see Section 4.3.3), female offspring of obese dams did not have impaired cardiac function as measured by *in vivo* echocardiography of the LV (Table 5.3). However in the female offspring of obese-exercised dams there were strikingly similar differences to the male offspring from the intervention group. Offspring of obese-exercised dams had increased LVPW at systole compared to offspring of control dams ($p< 0.01$). This caused an increased wall: lumen ratio at systole in these offspring ($p< 0.01$) (Table 5.3). The width at systole of the IVS and LVID were not affected. LV tracing measurements showed an increased ejection fraction and fractional shortening in female offspring born to obese-exercised dams (Table 5.3).

TABLE 5.3: *In vivo* cardiac function parameters. Data is presented mean ± SEM. *P* values calculated by one-way ANOVA. Letters represent differences between specified groups by Bonferroni post-hoc test ($p< 0.05$): [b] Control vs. Ob-Ex. Control $n= 9$, Obese $n= 7$ and Ob-Ex $n= 7$. For LVPW and wall: lumen ratio measurements; **1 *outlier*** detected in control group.

M mode dimensions	*Control*	*Obese*	*Ob-Ex*	*p value*
IVS; diastole (mm)	0.807 ± 0.028	0.886 ± 0.087	0.779 ± 0.043	0.396
LVID; diastole (mm)	3.56 ± 0.08	3.56 ± 0.07	3.52 ± 0.08	0.945
LVPW; diastole (mm)	0.75 ± 0.045	0.763 ± 0.063	0.81 ± 0.025	0.649
Wall:Lumen ratio; diastole	0.221 ± 0.013	0.215 ± 0.018	0.233 ± 0.010	0.510
IVS; systole (mm)	1.35 ± 0.05	1.42 ± 0.08	1.29 ± 0.04	0.308
LVID; systole (mm)	2.26 ± 0.09	2.18 ± 0.07	2.08 ± 0.09	0.321
LVPW; systole (mm)	1.03 ± 0.04	1.15 ± 0.10	1.35 ± 0.04[b]	**0.005**
Wall:Lumen ratio; systole	0.457 ± 0.026	0.534 ± 0.050	0.661 ± 0.041[b]	**0.005**
LV mass (mg)	92.3 ± 5.4	102.9 ± 8.3	95.4 ± 5.5	0.496
LV tracing measurements				
End diastolic diameter (mm)	3.60 ± 0.08	3.62 ± 0.06	3.54 ± 0.09	0.790
End diastolic volume (μl)	54.84 ± 2.98	54.52 ± 2.11	52.72 ± 3.19	0.871
End systolic diameter (mm)	2.24 ± 0.07	2.18 ± 0.05	2.04 ± 0.10	0.211
End systolic volume (μl)	17.15 ± 1.36	15.9 ± 0.87	13.82 ± 1.63	0.227
Stroke volume (μl)	37.71 ± 1.80	38.68 ± 1.63	38.9 ± 1.70	0.870
Heart rate (bpm)	471 ± 17	457 ± 10	460 ± 8	0.718
Cardiac output (ml/min)	17.86 ± 1.28	17.83 ± 0.66	17.92 ± 0.97	0.998
Ejection fraction (%)	69.02 ± 1.20	71.54 ± 0.97	74.33 ± 1.67[b]	**0.027**
Fractional shortening (%)	38.02 ± 0.92	40.00 ± 0.81	42.53 ± 1.43[b]	**0.022**

† LV= Left ventricle, IVS= Interventricular septum, LVID= LV internal diameter and LVPW= LV posterior wall.

5.4 Discussion

The aim of this chapter was to firstly determine the impact of maternal obesity exposure on the cardiovascular health of the female offspring and secondly to provide preliminary data into the impact of maternal exercise intervention on female offspring *in vivo* cardiovascular function. The findings revealed that, like male offspring, female offspring of obese dams had increased heart weight both in absolute terms and when expressed relative to bodyweight. However, unlike the male offspring where there was hypertrophy of the LV, the female offspring of obese dams had markers for RV hypertrophy and not LV hypertrophy. In keeping with the hypertensive phenotype of the male offspring of obese dams, the female offspring also had elevated SBP without any change in pulse rate and this was not corrected by the maternal exercise intervention. Consistent with a lack of LV cardiac hypertrophy, there were no changes in cardiac function when systolic *in vivo* function was assessed in the LV. The exercise intervention in the obese dam caused increased contractility in the hearts of the female offspring demonstrated by increased systolic wall: lumen, ejection fraction and fractional shortening.

5.4.1 Offspring body composition and serum analysis

An overall effect on bodyweight suggests a small reduction in bodyweight in the offspring of obese dams however when bodyweight was plotted individually at eight weeks of age there was no significant difference. Lactation data shown in Section 3.3.3 shows maternal obesity caused decreased bodyweight shortly after birth and an accelerated growth rate through the lactation period. This was not significantly altered with the exercise intervention. As it was not possible to separate by sex, both sexes were included in this analysis, sex differences in the growth profile of the pups after birth could have been concealed. An altered growth profile was continued post weaning with a suggestion that growth was reduced once females were weaned onto the postnatal control RM1 diet. Food intake measurements were not possible as for accurate data the same number of animals is required in every cage and in this experiment the numbers per cage varied between two and three.

As in male offspring, fat mass was increased in female offspring of obese dams with the fat mass diverging from seven weeks of age. Offspring of control dams did not appear to gain fat mass past this age but the offspring of obese dams continued to gain. This difference in fat mass was not present in eight week old animals after an overnight fast. Lean mass was

decreased by maternal diet with offspring of obese dams having reduced lean mass compared to offspring of control dams which was consistent with the reduction in bodyweight.

Plasma FFA levels were increased in the offspring of obese dams. FFAs are usually elevated in obesity, the increased adipose tissue mass releases more FFAs and also the clearance of FFAs could be reduced (Bjorntorp, Bergman and Varnauskas, 1969). Interestingly, elevated plasma FFAs has been associated with insulin resistance by inhibiting insulin stimulated glucose uptake (Boden *et al.*, 1994). Whole body insulin resistance did not appear to be present in the female offspring at this age as the serum fasting insulin was not increased; however there could be a selective insulin resistance in adipocytes and this should be assessed in the future. Furthermore this could suggest these offspring could be at increased risk of developing insulin resistance in later life.

The absence of hyperinsulinemia in the females is unexpected, since the male offspring of obese dams were hyperinsulinemic at the same age and females tend to be inherently more insulin resistant and have higher circulating insulin levels from birth (Murphy *et al.*, 2004; Manios *et al.*, 2014). A study utilising the same diet-induced model did not demonstrate hyperinsulinemia until three months of age that progressed to a diabetic phenotype at six months of age that suggested β-cell exhaustion (Samuelsson *et al.*, 2008). It is also important to note that the oestrous cycle of the female offspring was not monitored during the current study and this may have affected the results, as sex hormones are known to affect insulin sensitivity and glucose tolerance (Bailey and Matty, 1972).

5.4.2 Maternal obesity programmed RV but not LV hypertrophy in female offspring

As part of this project, the method for assessing cardiomyocyte cell area was optimised to become automated using new equipment and software purchased by the department during the course of the PhD. This has benefits in that this will reduce bias and also speed up the process of analysis. With the new method it was possible to image the entire WGA stained section enabling analysis to be performed in both ventricles, which was not possible at the time of doing the analysis on the male offspring. The relatively low analysis burden of utilising the HALO^{TM} software greatly improved the efficiency of this analysis. This was critical in identifying the crucial finding that the RV and not the LV was involved in the cardiac hypertrophy in females. Cardiomyocyte cell area demonstrated significant differences but only in the RV and not the LV.

An interim analysis of mid-cardiac wall widths and ventricle area was performed on the same sections used for the cardiomyocyte cell analysis. The limitation of this analysis is that this was not carried out over 10 representative and equally spaced heart sections across the whole heart. Therefore, volume calculations could not be carried out and a comprehensive analysis was not possible. Hopefully, however, it provides a valid snapshot of morphology changes in the mid-cardiac section of the hearts. It is not currently known if these changes are present when the whole heart is considered and should form part of the future work for this project.

Consistent with the increased heart weight recorded at *post mortem*, stereological analysis also revealed an increase in total area of the heart section. The effect on cardiomyocyte size in the RV was reinforced by the differences shown in the RV stereological measurements. Increased RV wall width and total RV area (includes wall and lumen) were demonstrated in the mid-cardiac sections from the offspring of obese dams. The RV area was also increased when expressed relative to the total area of that section suggesting a disproportionate increase in area of this ventricle. The increased RV wall width suggests the increased area could be due to RV wall thickening, without a corresponding change in the lumen. The reduction in the LV: RV area ratio demonstrated a shift in the relative size contribution between the ventricles; where the RV was larger in the female offspring of obese dams. The RV morphological changes occurred without any significant changes in the corresponding LV measurements. This asymmetric growth in the RV could lead to LV dysfunction due to the mechanical interaction between the ventricles (Slinker, Chagas and Glantz, 1987), in fact deficits in diastolic function have been demonstrated previously in a rodent model of RV hypertrophy (Lamberts *et al.*, 2007). The presence of LV contractile dysfunction was not supported in the LV systolic cardiac measurements.

Previous work in the laboratory has assessed the time course of cardiac hypertrophy in the male offspring. Hypertrophy was present from three weeks of age and an accelerated cardiac growth profile was present in the male offspring of obese dams. Interestingly biventricular cardiac growth was present up until five weeks of age before the LV growth dominated (Blackmore *et al.*, 2014). At 12 weeks of age, the LV hypertrophy resolves suggesting a plateau in cardiac growth between 8-12 weeks of age in the male offspring of obese dams. This might suggest a different cardiac growth profile in the females and therefore LV hypertrophy may develop as the animal ages. The male offspring of obese dams appear to display hypertrophy at a more advanced stage then the female offspring. It would be

interesting to assess cardiac hypertrophy at younger and older ages in the females to establish a time course of cardiac growth.

These structural changes were accompanied by increased expression of *Nppa* and *Myh*7, two fetal genes known to be re-expressed in pathological, but not physiological, cardiac hypertrophy (Nakamura and Sadoshima, 2018). This expression was measured in whole ventricular tissue and has not been analysed in each ventricle separately. This re-expression was probably driven by changes in the RV and the expression profile may have been more dramatic if the RV was assessed alone. Rockman and colleagues demonstrated increased *Nppa* in a pulmonary aorta banded model of RV hypertrophy (Rockman *et al.*, 1994). The re-expression of these genes is also present in the RV of a mouse model of right sided hypertrophy (Bartelds *et al.*, 2011).

The cardiac hypertrophy was not accompanied by cardiac fibrosis or deficits in oxidative stress response and this might suggest this hypertrophy may not be truly pathogenic in nature. The fibrosis, however, was analysed across the whole heart section with 10 random grids selected for quantification, therefore each ventricle was not analysed separately. This may have revealed differences in fibrosis isolated to the RV. RV maladaptive remodelling is associated with cardiac fibrosis and failure of antioxidant defence, this was not present in the female offspring when expression was assessed in both ventricles suggesting that there has not been progression to this stage of disease. However, if expression was just assessed in the RV, significant differences in key oxidative stress markers may be present.

5.4.3 Possible mechanisms for RV hypertrophy

Thickening of the left and right ventricle is commonly associated with systemic and pulmonary hypertension, respectively. However, evidence from human studies provides evidence that remodelling of the RV wall may also occur indirectly as a consequence of systemic hypertension. Patients with essential hypertension had a RV wall two-fold thicker than their normotensive counterparts (Nunez *et al.*, 1987). In a more recent study, 1 in 5 hypertensive patients developed biventricular cardiac hypertrophy (Cuspidi *et al.*, 2014).

Systemic hypertension has been shown to be associated with elevated pulmonary arterial pressures (Oliveri *et al.*, 1978). RV hypertrophy in systemic hypertension has been attributed to LV diastolic dysfunction acting via pulmonary venous hypertension and increased RV end-diastolic pressure (Akintunde *et al.*, 2010; Cuspidi *et al.*, 2014). RV hypertrophy could be a

consequence of pulmonary vascular resistance which in turn causes RV pressure overload (Asosingh and Erzurum, 2018). Notably, babies born to obese women are at an increased risk of respiratory complications at birth and in childhood (Hernandez-Diaz *et al.*, 2007; Lock *et al.*, 2013). In a rodent model of calorie restriction during gestation combined with hypoxic stress later in life, offspring developed pulmonary hypertension and RV cardiac hypertrophy. Programming of this adverse phenotype in offspring was associated with epigenetic mechanisms through alterations in lung DNA methylation (Rexhaj and Bloch, 2011). The future work should assess the cause of the RV hypertrophy. Right ventricular systolic pressure as a proxy for the pulmonary artery pressure can be measured by right heart catheterisation.

The fat mass of the female offspring of obese dams starts to diverge from seven weeks of age and may indicate that these animals have a predisposition to becoming obese in the future. Obesity, in the absence of traditional cardiovascular risk factors has been linked to RV hypertrophy. It has been shown that, with increasing BMI, women have proportionally greater RV hypertrophy independently of LV hypertrophy (Rider *et al.*, 2014). This can be explained either by the fact that cytokines, adipokines and growth factors have a greater impact on the RV in females then in males or that sex hormones are somehow responsible. Furthering the hypothesis that sex has an effect on RV remodelling is a study that has demonstrated a stronger influence of metabolic syndrome on RV remodelling in females then in males (Tadic, Ivanovic and Grozdic, 2011).

In Chapter 4, a possible mechanism for the observed LV cardiac hypertrophy was suggested to be hyperinsulinemia which is present in the mother and also present in the male offspring of obese dams at eight weeks of age. In contrast the female offspring of obese dams are not hyperinsulinemic at this age but will still have been exposed to maternal hyperinsulinemia during the fetal and neonatal period (Section 3.3.4). It will be important in future studies to assess insulin at different time points, insulin levels could have been high in females before birth and during lactation period. The initial insult may not need to be maintained to see an impact on heart growth. Equally, another explanation is that the suggested mechanism may be critical in driving the LV sided hypertrophy and a different mechanism is involved in the RV hypertrophy. As the female ages, hyperinsulinemia might develop and the LV hypertrophy could develop later than in the male offspring.

5.4.4 Elevated SBP and its impact on cardiac parameters

SBP was elevated in the female offspring of obese dams and may share a common programming mechanism as the same was observed in the male offspring (see Section 4.3.3). Again, the exercise intervention did not modify this offspring outcome reinforcing the suggestion that this pathway involves a programming factor in the mother that is not modifiable by the exercise intervention (discussed in Section 4.4.4).

Data from the male offspring suggested a disparity in the development of LV hypertrophy and hypertension as offspring of obese-exercised dams did not have both outcomes despite the effects of them being linked. Females born to obese dams also show hypertensive hearts that did not have hypertrophy in the LV. The reason for this is unclear, although hypertension will inevitably contribute to LV hypertrophy it is unlikely to be causative in these offspring. A study of patients with hypertension observed that more female patients had hypertension without LV hypertrophy then those who were hypertensive with accompanying LV hypertrophy; the same effect was not seen in the males (Jakovljevic *et al.*, 2010). This could suggest that hypertensive females are more protected from LV hypertrophy.

5.4.5 Protective effect on cardiac function in female offspring

Unlike the male offspring, LV hypertrophy and systolic dysfunction were not present in the female offspring, this shows a sex difference in the offspring's cardiovascular response to maternal obesity. There was no cardiac dysfunction despite increased afterload from elevated SBP; this indicates a gender difference in the response of the heart to a sustained elevated level of myocardial stress. Women often display preserved systolic function in situations where males have shown systolic dysfunction (Hayward, Kalnins and Kelly, 2001) and this could be the reason that no cardiac dysfunction was detected in the female offspring of obese dams. In a transverse aortic constriction model, female rats developed increased SBP but did not progress to heart failure unlike males who developed ventricular dilatation, increased wall stress and dysfunction (Douglas *et al.*, 1998).

The most highly discussed mediator of sex differences in cardiac physiology is estrogen. Estrogen has been shown to have anti-hypertrophic properties. In both volume and pressure overload models, ovariectomized females developed significantly more cardiac hypertrophy than intact females (Brower, Gardner and Janicki, 2003; Bhuiyan, Shioda and Fukunaga, 2007). *In vitro* experiments in neonatal rat ventricular cardiomyocytes have shown that

estrogen increases the expression of *Nppa* which antagonises the development of LV hypertrophy (Babiker *et al.*, 2004).

With the echocardiographic method used in this thesis, the diastolic measurements could not easily be made and this remains a limitation of this study. Future work should assess the LV diastolic function as RV hypertrophy has been associated with LV diastolic dysfunction (Akintunde *et al.*, 2010; Rider *et al.*, 2014). It is more technically difficult to assess the RV function by echocardiography but for future studies an importance should be placed on achieving this. As RV hypertrophy is an independent risk factor for heart failure and cardiovascular mortality (Haddad *et al.*, 2008) it is important to get a full assessment of cardiac function in the animals.

5.4.6 Effect of maternal exercise on offspring cardiac function

Maternal obesity has no effect on cardiac function in female offspring at eight weeks of age but there is a disparity in how the hearts of the female offspring with elevated SBP were behaving, depending on if the obese mother was exercised during pregnancy. When maternal obesity was combined with maternal exercise intervention during pregnancy, an increased heart contractility was seen compared to offspring of control dams, as shown by increased systolic wall: lumen ratio, ejection fraction and fractional shortening. The cardiac contractility was even greater than offspring of control dams and was despite the hemodynamic stress of the elevated SBP that was also present in these females. This might be a direct compensatory response to elevated SBP. The offspring of obese-exercised dams appear to have been far more effective at responding to an increased afterload and this was true for both sexes, but unlike the males the increased afterload in the offspring of obese dams did not cause cardiac dysfunction. The increase in inotropy demonstrated in female offspring of obese-exercised dams could be sympathetic activated, and this should be explored in the female offspring.

Exercise during pregnancy has been shown to elicit a training response in the fetal heart by lowering fetal HR and increasing HR variability (May *et al.*, 2012); these changes have then been shown to be carried on into the infant period (May *et al.*, 2010). These cardiac adaptations are seen in adults as a response to exercise and are associated with a lowering of CVD risk (Shephard and Balady, 1999). It would be very insightful to determine if maternal exercise during pregnancy could confer increased cardiac contractility in offspring hearts when elevated SBP is not also present. This would determine if this was a directly programmed mechanism in these hearts or a compensatory response to maintain cardiac

output. This could perhaps be elucidated by carrying out a fourth experimental group where control dams are exercised.

5.6.7 Conclusions

Maternal obesity had both similar and diverse effects on the cardiovascular outcomes of the female offspring when compared to the male offspring. Offspring hypertension was programmed in the offspring of obese dams irrespective of sex. However sex differences were present in the programmed effects on cardiac hypertrophy and function. In the female offspring of obese dams there was no effect on the LV however there was an impact on the RV. RV hypertrophy was demonstrated by increased RV wall width and area, increased cardiomyocyte cell area and re-expression of fetal genes. Further work is needed to determine if this hypertrophy is truly pathological in nature by evaluating RV function. This work highlights the importance of carrying out studies in both sexes as the developmental programming effects are very different.

The maternal exercise intervention did not prevent the programmed offspring hypertension and produced a similar response compared to the male offspring through increased cardiac contractility. The exercise in the obese dam during pregnancy programmed a response that has similarities to the normal cardiac response to exercise, despite the offspring never having exercised themselves.

5.6.8 Summary of key findings

Results of this chapter have shown:

- The female offspring of obese dams at eight weeks of age were smaller (reduced bodyweight and lean mass) but had increased fat mass.
- Maternal obesity caused RV hypertrophy in the female offspring demonstrated through increased RV wall width and area, increased cardiomyocyte area and re-expression of fetal genes. This hypertrophy was not accompanied by changes in cardiac fibrosis and oxidative stress.
- The female offspring of obese dams were hypertensive but unlike the male offspring this was not associated with any systolic cardiac dysfunction in the LV.
- Maternal exercise intervention caused increased cardiac contractility in the female offspring despite the elevated SBP; these results mirrored those in the male offspring.

6. Immediate consequences of an obese pregnancy on the fetal heart

6.1 Introduction

6.1.1 *In utero* cardiac development

The heart is the first organ to form and function in the developing fetus. In the mouse, the heart begins as a single beating heart tube at E8.0 but then develops into a four-chambered heart with functioning valves and a separate outflow tract. Primitive circulation is set up from E8.5 and ventricular morphogenesis occurs between E11-E16 (Savolainen, Foley and Elmore, 2009). Cardiac morphogenesis and the formation of the four chambered heart in mice is comparable to that of humans, however in mice the developmental events happen over a shorter gestational window (Wessels and Sedmera, 2003). In the human, first heart contractions happen at around three weeks after conception, with completion of ventricular septation at around nine weeks Systematic comparative analysis of mouse and human fetal cardiovascular development has been carried out and demonstrated that the cardiac developmental sequences are comparable, with only minor differences in atrial and venous morphology. These differences include the mouse fetal heart having a thicker and more muscular atrioventricular septum, and larger, more prominent atrial appendages (Krishnan *et al.*, 2014).

Fetal circulation is distinct from the system in the adult, this is true in both mouse and human development (Figure 6.1). Oxygenated blood from the placenta enters the right atrium and is shunted to the left atrium through an inter atria channel, called the foramen ovale, and then into the LV. Blood is pumped out through the aorta into the body, where the now deoxygenated blood is returned through the superior vena cava to the right atrium and then on to the RV. The blood is pumped out of the pulmonary artery, however since the lungs are not yet fully functional, high pulmonary resistance forces the majority of the blood into the descending aorta through a shunt called the ductus arteriosus (Savolainen, Foley and Elmore, 2009). Both ventricles eject blood in parallel into the systemic circulation; the LV directs the most oxygenated blood towards the heart and brain, and the RV ejects the less oxygenated blood to the lower body and placenta (Rizzo, Arduini and Romanini, 1992). Separation of the pulmonary and systemic circulation does not happen until after birth, when there is a

transition from fetal dependence on the placenta for oxygenated blood to self-oxygenation by the lungs. This important developmental event is achieved through the closure of the foramen ovale and the ductus arteriosus (Savolainen, Foley and Elmore, 2009).

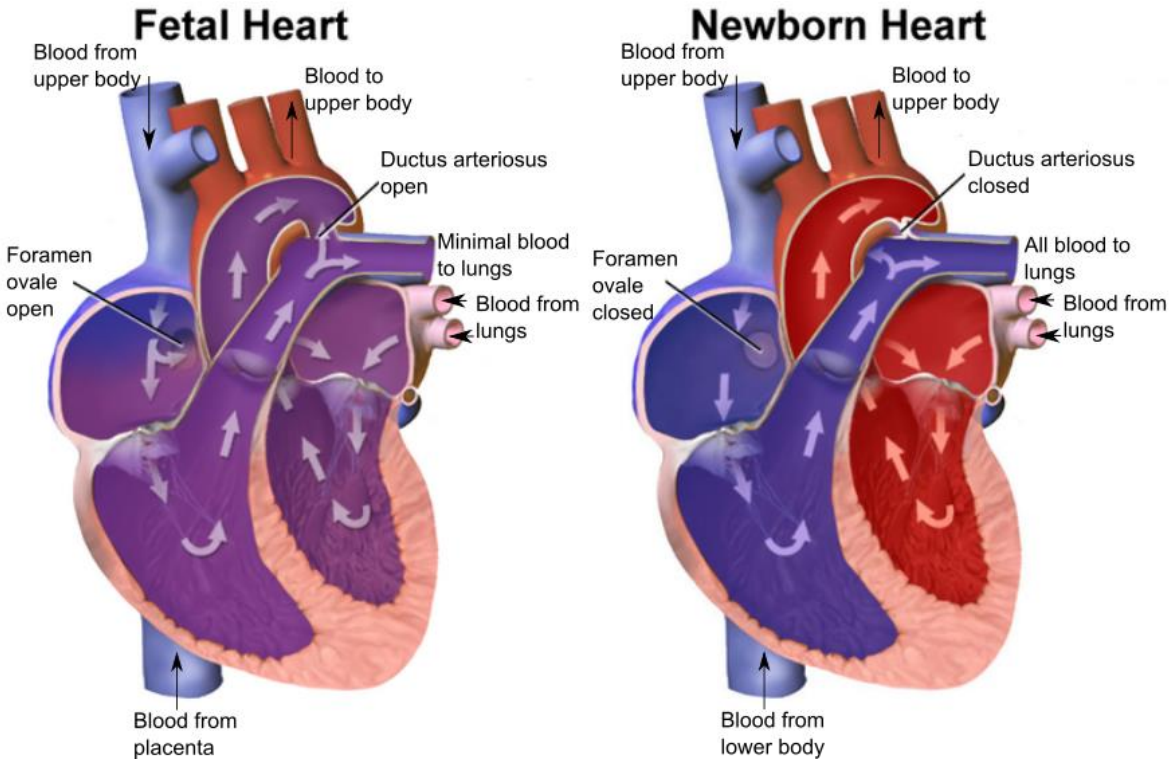


FIGURE 6.1: Differences in the anatomy and function of the human fetal heart. Modified with the permission [CC BY-SA] of Bruce Blaus, Blausen.com. Medical gallery of Blausen Medical 2014.

Proliferation of cardiomyocytes is critical in supporting the increasing hemodynamic load during embryonic development. Cardiomyocyte proliferation must be regulated for normal morphogenesis to occur and will determine appropriate heart size. Apoptosis is equally important for developmental remodelling of the heart and has been shown to be critical in the development of the embryonic outflow tract, cardiac valves, and coronary vasculature (Fisher, Langille and Srivastava, 2000). The number of viable cardiomyocytes dictates cardiac function and is determined in early life, with negligible increases after that. In general, cell proliferation and hypertrophy allows cardiac growth before and after birth respectively in the human heart. In the rodent heart, the transition of proliferative and hyperplastic growth of mononucleated cells to hypertrophic growth of binucleated cells, and terminal differentiation of cardiomyocytes, takes place within the first two week after birth (Clubb and Bishop, 1984; Soonpaa *et al.*, 1996).

Regulation of cardiomyocyte endowment in fetal life is critically important for the future cardiovascular health of that individual. Due to the inability in later life for cardiomyocytes to proliferate, the number of cardiomyocytes at birth will be similar to the number available for life (Thornburg *et al.*, 2011). Cardiomyocyte proliferation is under the regulation of mediators such as insulin-like growth factor (Sundgren, Giraud, Schultz, *et al.*, 2003), angiotensin II (Sundgren, Giraud, Stork, *et al.*, 2003), and corticosterone (Giraud *et al.*, 2006), as well as mechanical stimuli such as arterial pressure load (Giraud *et al.*, 2005). Suppressors of cardiomyocyte proliferation include triiodothyronine (Chattergoon, Giraud and Thornburg, 2007), atrial natriuretic peptide (O'Tierney, Chattergoon, *et al.*, 2010), and reduced cardiac systolic load (O'Tierney, Anderson, *et al.*, 2010). Poorly controlled cardiomyocyte development will most likely lead to cardiac dysfunction, for example in growth-restricted animal models, a reduced number of cardiomyocytes might leave the heart vulnerable in conditions of high workload (Porrello, Widdop and Delbridge, 2008)

6.1.2 Intrauterine exposures that impact on fetal development

Hypoxia

Hypoxia is a major fetal stressor and can occur in pre-eclampsia, placental insufficiency, and nicotine exposure during pregnancy (Paradis, Gay and Zhang, 2014). Restricted oxygen supply is necessary for normal intrauterine organ development, however excessive or severe hypoxia is damaging (Tong *et al.*, 2011). The fetus can initially adapt to hypoxia by increasing the blood supply to the brain, heart and upper body, while decreasing perfusion to the lower body. This redistribution in blood flow has consequences in the heart; cerebral vasodilation leads to decreased LV afterload and the opposing systemic vasoconstriction increases RV afterload (Al-Ghazali *et al.*, 1989). In rodents, the heart is particularly vulnerable to stressors such as hypoxia, during late fetal development and early postnatal life when it undergoes rapid growth and maturation (Louey and Thornburg, 2005).

Hypoxia exposure during pregnancy in a rat causes increased heart to bodyweight ratio in the fetus and this is accompanied by decreased LV wall thickness (Tong *et al.*, 2011). Hypoxia inhibits proliferation of fetal rat cardiomyocytes (Tong *et al.*, 2013) and increases the cell size and percentage of binucleated cardiomyocytes in fetal rat hearts (Bae *et al.*, 2003). Fetal hypoxia causes a premature exit of the cell cycle, and if this deficit in proliferation leads to fewer but larger cardiomyocytes then this will predispose the individual to cardiac

hypertrophy. Cardiac remodelling was seen in both fetal and neonatal rat hearts when exposed to hypoxia during pregnancy (Tong *et al.*, 2011).

Glucocorticoids

Glucocorticoids are a class of hormone that are essential for normal lung and cardiovascular system development and in particular maturation. Glucocorticoids are administered to preterm babies to promote lung development, but although there is short-term benefits for immediate survival, there are also possible long-term side effects (Bolt *et al.*, 2001). Fetal sheep infused with cortisol have increased proliferation of cardiomyocyte without an increase in size. There were no differences in the percentage of binucleated cells, therefore the differentiation/maturation of the cardiomyocytes was unaffected and instead there was hyperplastic growth (Giraud *et al.*, 2006). A fetal rat low-dose dexamethasone model also showed increased proliferation and a delay in heart maturation (Torres *et al.*, 1997).

IUGR

IUGR occurs in a range of conditions (e.g. inadequate nutrition, placental insufficiency and hypoxia) and is defined as inadequate growth. A sheep model of placental insufficiency using umbilicoplacental embolization showed reduced heart and bodyweight with no difference in heart to bodyweight ratio. Proliferation and maturation of the cardiomyocytes was suppressed; alongside the finding that the smaller hearts did not have reductions in cardiomyocyte size, thus suggesting a deficit in working cardiomyocytes (Louey *et al.*, 2007).

Maternal protein restriction in rats also resulted in pups of lower birthweight and reduced heart size. The number and size of cardiomyocytes were reduced in one day old pups without any changes in the fraction of binucleated cells (Corstius *et al.*, 2005). The burden of mechanical stresses and contractile force generation was consequently shared between fewer cardiomyocytes, therefore as a compensatory mechanism to normalize wall stress there was hypertrophy of the chamber wall accompanied by cardiomyocyte enlargement (Roberts, Frias and Grove, 2015).

Maternal obesity

Maternal obesity has previously been associated with an increased risk of congenital heart malformations in humans (Mikhail, Walker and Mittendorf, 2002; Mills *et al.*, 2010). Assessment of fetal anatomy, in particular identifying fetal heart abnormalities, is far more limited in obese women due to poor ultrasound visualisation, and this can have adverse

consequences (Hendler *et al.*, 2005). The missed diagnosis of congenital heart problems greatly increases the risk of death, as proper and timely treatment cannot be administered (Chang, Gurvitz and Rodriguez, 2008).

A study of pregnant women was undertaken to assess the effect of maternal obesity on fetal cardiac function through fetal echocardiography. The obese pregnant women had increased SBP and resting HR. At 14 weeks of gestation, fetal echocardiography demonstrated decreased global strain rate of the left and right ventricle in fetal hearts, which is a measure of reduced systolic function (Ingul *et al.*, 2016).

An overfeeding sheep model of maternal obesity caused increased bodyweight and LV weight. Cardiomyocytes had increased length, and decreased peak shortening and sarcomere length. Ca^{2+} homeostasis, which is critical for normal cardiac contraction, was disrupted and showed increased resting and peak Ca^{2+} in both LV and RV cardiomyocytes (Wang *et al.*, 2018).

6.1.3 Aims of chapter

The primary aim of the chapter was to assess the immediate consequences of maternal diet-induced obesity on the morphology of the fetal heart in both sexes. The impact of the maternal exercise intervention in the obese dam was also to be assessed. The second aim was to assess if oxidative stress is present in the fetal heart of obese dams.

6.2 Methods

6.2.1 E19 tissue collection

On embryonic day 19, dams were killed by rising CO_2 concentration and fetuses were dissected. Fetuses were sexed by visual inspection for testes, and individual bodyweights were recorded. Hearts were collected and snap frozen for MDA assay. Torsos (male and female) were immersion-fixed in formalin for stereological analysis. This was performed by Dr Denise Fernandez-Twinn and Dr Heather Blackmore.

6.2.2 Stereological assessment of E19 heart

Immersion-fixed whole E19 torsos were processed and the heart was exhaustively sectioned in the coronal plane at 10 µm. Due to the small size of the E19 heart (especially following processing) it was not possible to manually align the isolated heart before embedding, therefore whole torsos were aligned and fixed. The fetal torsos themselves aligned the heart,

and this allowed for a more successful embedding and serial sectioning process. Slides were chosen for staining using a systematic method, a total of 10 equally spaced sections throughout the heart were required for analysis. Firstly, the number of sections taken for each torso's heart was counted. The number of sections required between each stained section was calculated by dividing the total number of sections by 10. For example, for a heart cut into 150 sections, a stained section at every 15th interval was analysed. The chosen heart sections were stained with haematoxylin and eosin, and imaged on the Slide Scanner Axio Scan Z1 (Zeiss, Germany) using a 20x objective. A previously published stereological method was utilised (Blackmore *et al.*, 2014). Images were exported in TIFF format ensuring that a scale bar was added. Images were then analysed using FIJI software. Ventricle area was assessed by superimposing crosses onto the image where the area of the cross was known (previously described in Section 5.2.2). The plugin (available on FIJI) called Cell counter was used to count the number of crosses falling on the LV, LL, RV, RL and IVS. This was analysed over 10 sections across the whole heart. The Cavalieri principle was then used to calculate area and volume of ventricles and lumens:

$Area\ (mm^2) = A(p) \times \sum p$, *A(p) = known area /point.* Σp = *Sum of each point counted.*

$Volume\ (mm^3) = Area\ (mm^2) \times thickness\ of\ section\ (mm) \times total\ no. of\ sections$

Total LV area was considered to be the sum of the LVPW and the IVS area.

6.2.3 Lipid peroxidation MDA assay

Malondialdehyde (MDA) is an end product of lipid peroxidation and is therefore a good measure of oxidative stress. MDA was measured in E19 heart tissue through the use of a lipid peroxidation fluorometric assay kit (Abcam, UK). The protocol was adapted to account for the low tissue weight. The tissue was homogenised by hand using the lysis buffer provided and a micropestle (Sigma-Aldrich, USA). The MDA present in the prepared lysates reacted with thiobarbituric acid and formed an adduct that was then measured fluorometrically at an excitation/emission wavelength of 532/553 nm. The level of MDA (nmol/well) was calculated through the use of a standard curve of known MDA concentrations. This was then normalised per mg of heart tissue.

6.3 Results

6.3.1 Fetal bodyweight at E19

Fetal weights were measured at the point of *post mortem*. There was no effect of sex on fetal weight however there was a significant effect of maternal lifestyle. Maternal obesity decreased fetal weight in both sexes and this was not corrected by the maternal exercise intervention (Figure 6.2).

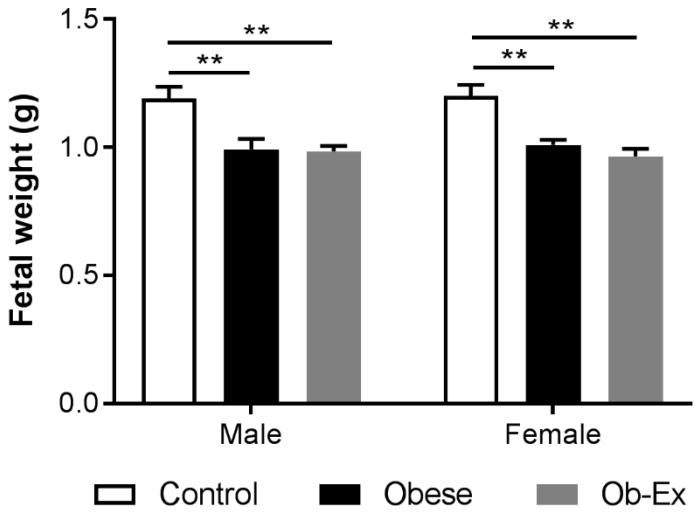


FIGURE 6.2: Fetal weights at E19. Male: Control *n*= 5, Obese *n*= 6 and Ob-Ex *n*= 4. Female: Control *n*= 5, Obese *n*= 6 and Ob-Ex *n*= 3. Two-way ANOVA with Bonferroni post-hoc test; ** $p< 0.01$.

6.3.2 Fetal heart alignment

Unfortunately, there were natural differences in the heart alignments between the individual fetal torsos which reflects biological heterogeneity in how the heart lies in the chest cavity. This was a major limitation encountered in this study which was not anticipated when designing the experiments. In the males, roughly one third of all analysed fetal hearts were at a different alignment, with the apex of the heart pointing upwards towards the sternum as opposed to lying flatter in the chest cavity. This was not confined to one experimental group, appearing equally between all maternal groups. This meant the heart appeared 'twisted' with the RV not being well represented in the first few (of the 10) sections but becomes dominant in the later sections. Figure 6.3 illustrates representative mid-cardiac sections showing the two alignments.

The alignment of the heart within the chest cavity also appeared to be influenced by sex. In the females, all of those analysed appeared to have the 'twisted' heart alignment. In support of

this, when performing echocardiography analysis in the female offspring at 8 weeks of age (for Chapter 5), it was noted that the transducer had to be positioned differently compared to the males to achieve the parasternal axis view needed for analysis. Due to these sex differences it was not possible to directly compare stereological differences between the sexes at E19.

Ventricular wall width analysis of the mid-cardiac section was not carried out, as this is highly dependent on correct alignment and the results would not be meaningful. Additionally, as shown in Figure 6.3, the RV wall is under-represented in the mid-cardiac section of the 'twisted' alignment. Unless identified as a Grubbs statistical outlier ($p< 0.05$), all hearts were included in the area/volume analysis, as this was considered most stringent and least likely to add bias. Those with the 'twisted' alignment are shown as red data points in the scatter plots of this results section. Despite the different heart alignments, total heart and ventricular area/volumes appear fairly similar within groups. The alignment had most impact on the lumen measurements.

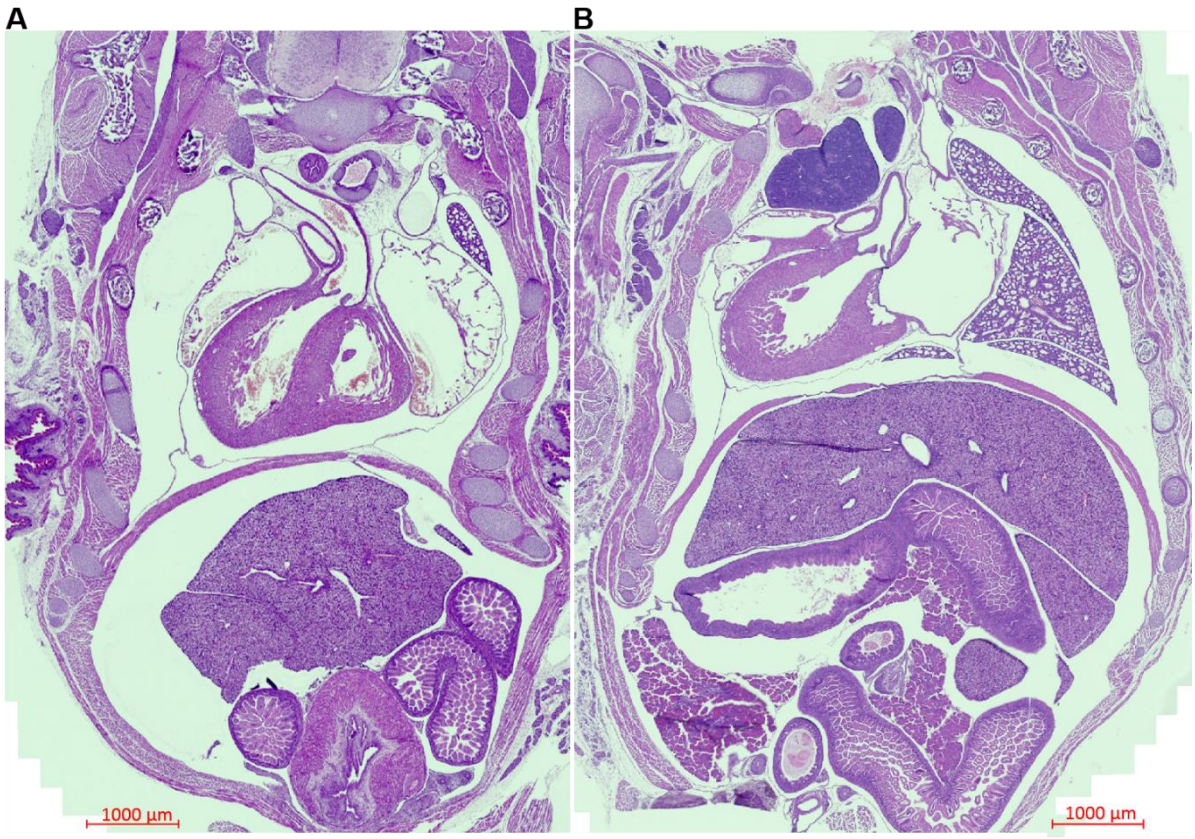


FIGURE 6.3: Representative images showing the two cardiac alignments. Images show mid-cardiac sections of haematoxylin and eosin stained torsos. A) Standard alignment B) 'twisted' alignment where heart points up towards sternum.

6.3.3 E19 total heart area and volume

Males

Absolute total area was not altered between groups in the male fetal hearts, and similarly heart volume was also unaltered (Figure 6.4A+B). When expressed relative to fetal weight, heart area continued to be unchanged (Figure 6.4C), however heart volume expressed relative to fetal weight was increased in the fetuses of obesogenic diet-fed dams (Figure 6.4D). The relative contributions to heart volume of the two ventricles was unaltered between groups as shown by the calculated LV: RV volume ratio (Control, 1.80 ± 0.05; Obese, 2.07 ± 0.11; Ob-Ex, 1.88 ± 0.05; p= 0.074).

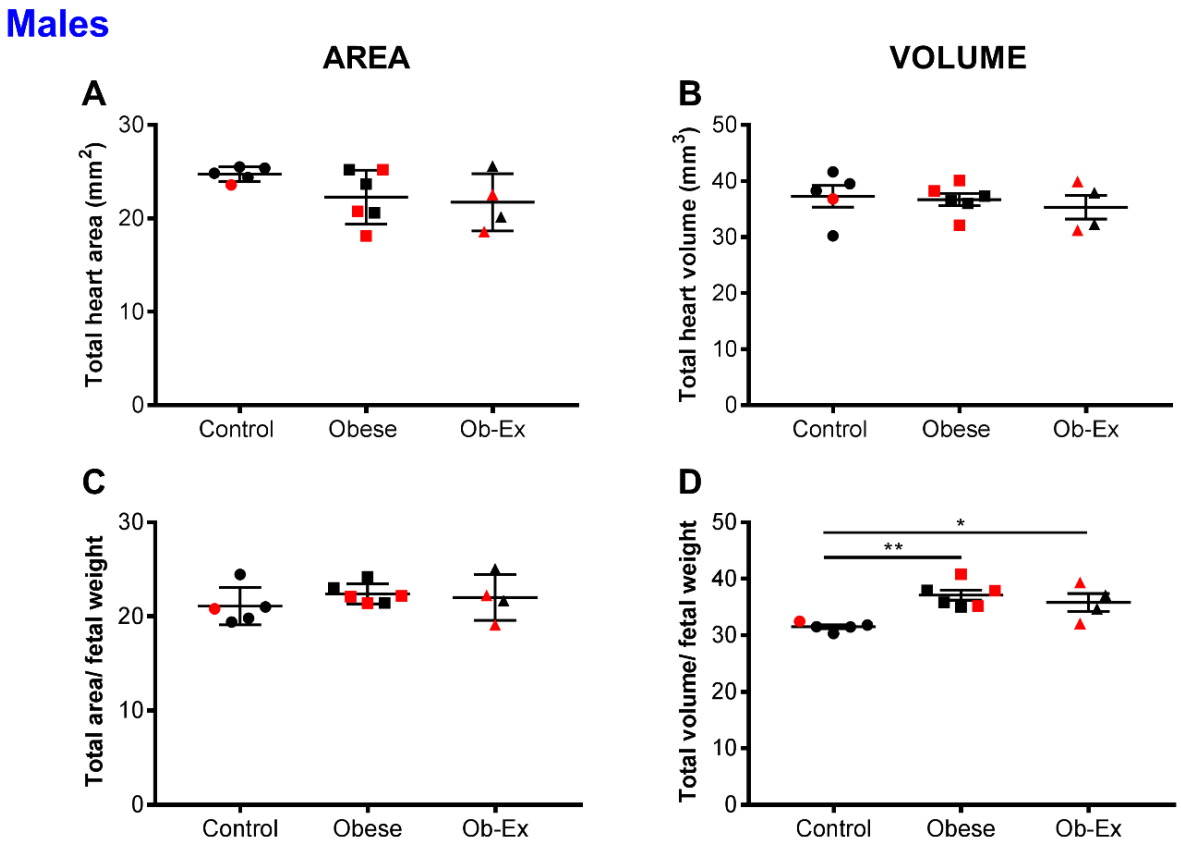


FIGURE 6.4: Total area and volume of male fetal heart. A) Total heart area B) Total heart volume C) Total heart area relative to fetal weight and D) Total heart volume relative to fetal weight. Red data points are those analysed hearts with 'twisted' alignment. Control n= 5, Obese n= 6 and Ob-Ex n= 4. One-way ANOVA with Bonferroni post-hoc test; * p< 0.05 and ** p< 0.01.

Females

In the female hearts, area was increased in the fetuses of obese-exercised dams compared to fetuses in the non-exercised obese dams (Figure 6.5A). This did not result in any changes in

the volume of the female hearts between groups (Figure 6.5B). There was a significant difference in the heart area relative to fetal weight in the fetuses of obese-exercised dams compared to both the other groups (Figure 6.5C). Again, heart volume was not altered even with normalisation to fetal weight (Figure 6.5D). An unchanged LV: RV volume ratio demonstrated no differences in the relative volume of the two ventricles (Control, 1.29 ± 0.03; Obese, 1.27 ± 0.04; Ob-Ex 1.21 ± 0.10; p= 0.550).

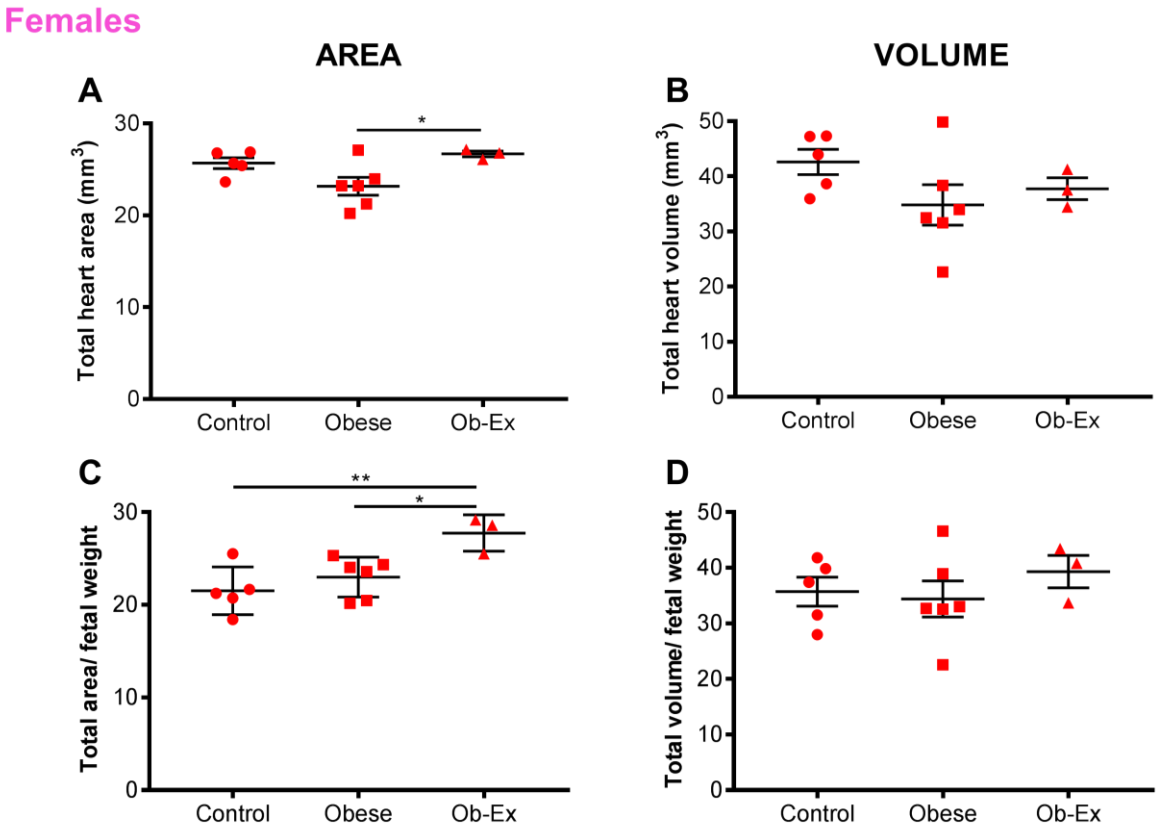


FIGURE 6.5: Total area and volume of female fetal heart. A) Total heart area B) Total heart volume C) Total heart area relative to fetal weight and D) Total heart volume relative to fetal weight. Red data points are those analysed hearts with 'twisted' alignment. Control n= 5, Obese n= 6 and Ob-Ex n= 3. One-way ANOVA with Bonferroni post-hoc test; * p< 0.05 and ** p< 0.01.

6.3.4 Area and volume: left side of the heart

Males

LV Area was decreased in the hearts of male fetuses of obese and obese-exercised dams (Figure 6.6A). There was no significant difference in LV volume (Figure 6.6B) or LV volume relative to fetal weight (Figure 6.6C). There was a significant increase in LL area in male fetuses of obese dams and the increase in fetuses of obese-exercised dams was not significant (Figure 6.6D). LL volume was increased in the male fetuses of both obese dams and obese-

exercised dams (Figure 6.6E). LL volume expressed relative to fetal weight was increased in male fetuses of obese dams but the increase in the obese-exercised dams was not significant (Figure 6.6F). The LV: LL volume ratio in the male fetal hearts showed significant differences (Control, 25.37 ± 5.57; Obese, 5.94 ± 1.15; Ob-Ex, 7.32 ± 2.94; p= 0.004); with post-hoc testing revealing a significant decrease in fetuses of obese and obese-exercised dams compared to fetuses of control dams.

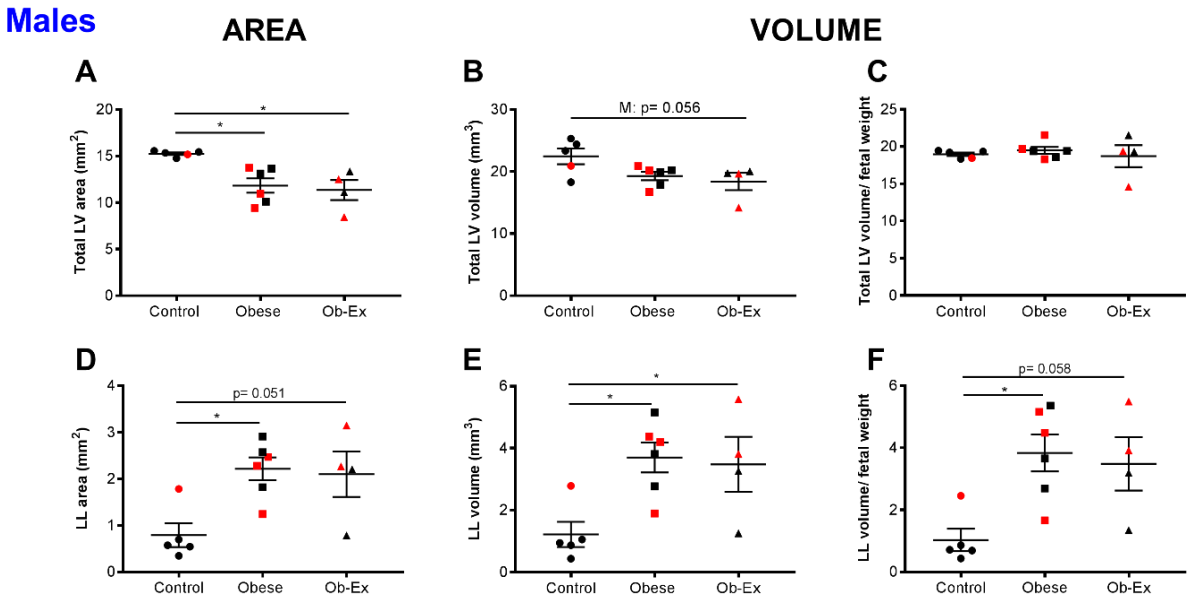


FIGURE 6.6: Area and volume of LV and LL in male E19 hearts. A) Total LV area B) Total LV volume C) Total LV volume relative to fetal weight D) LL area E) LL volume and F) LL volume relative to fetal weight. Red data points are those analysed hearts with 'twisted' alignment. Control n= 5, Obese n= 6 and Ob-Ex n= 4. One-way ANOVA with Bonferroni post-hoc test; * p< 0.05 and M: overall effect of maternal lifestyle.

Females

There was a decrease in the LV area and volume in female fetuses of obese dams (Figure 6.7A+B). There was no significant reduction in LV volume in the female fetuses of obese-exercised dams (Figure 6.7B). These differences were not present if LV volume was expressed relative to fetal weight (Figure 6.7C). There were no significant differences in the area or volume of the LL in the female fetal heart (Figure 6.7D-F). The LV: LL volume ratio in the females showed no significant differences between groups (Control, 11.52 ± 3.70; Obese, 8.59 ± 2.85; Ob-Ex, 4.92 ± 1.27; p= 0.450).

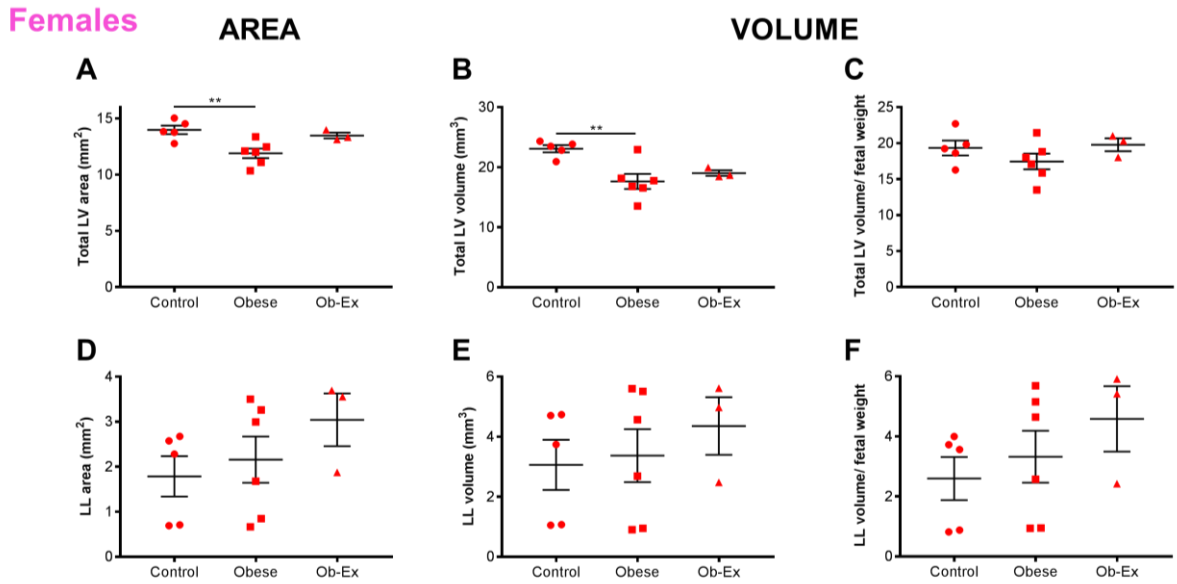


FIGURE 6.7: Area and volume of LV and LL in female E19 hearts. A) Total LV area B) Total LV volume C) Total LV volume relative to fetal weight D) LL area E) LL volume and F) LL volume relative to fetal weight. Red data points are those analysed hearts with 'twisted' alignment. Control *n*= 5, Obese *n*= 6 and Ob-Ex *n*= 3. One-way ANOVA with Bonferroni post-hoc test; ** $p< 0.01$.

6.3.5 Area and volume: right side of the heart

Males

In the male E19 fetal hearts, RV area and volume were decreased in the groups from obesogenic diet-fed dams when expressed as absolute values (Figure 6.8A+B); however, when expressed relative to fetal weight the difference was not present (Figure 6.8C). RL area and volume was increased in the hearts of fetuses of obese and obese-exercised dams (Figure 6.8D+E). Unlike the RV, the difference in RL volume as a consequence of maternal obesity was maintained when normalised to fetal weight (Figure 6.8F). The RV: RL volume ratio revealed statistical differences (Control, 14.17 ± 3.489; Obese, 2.59 ± 2.59; Ob-Ex 4.151 ± 1.974; $p= 0.006$); with post-hoc testing revealing a significant decrease in fetuses of obese ($p< 0.01$) and obese-exercised ($p< 0.05$) dams compared to fetuses of control dams.

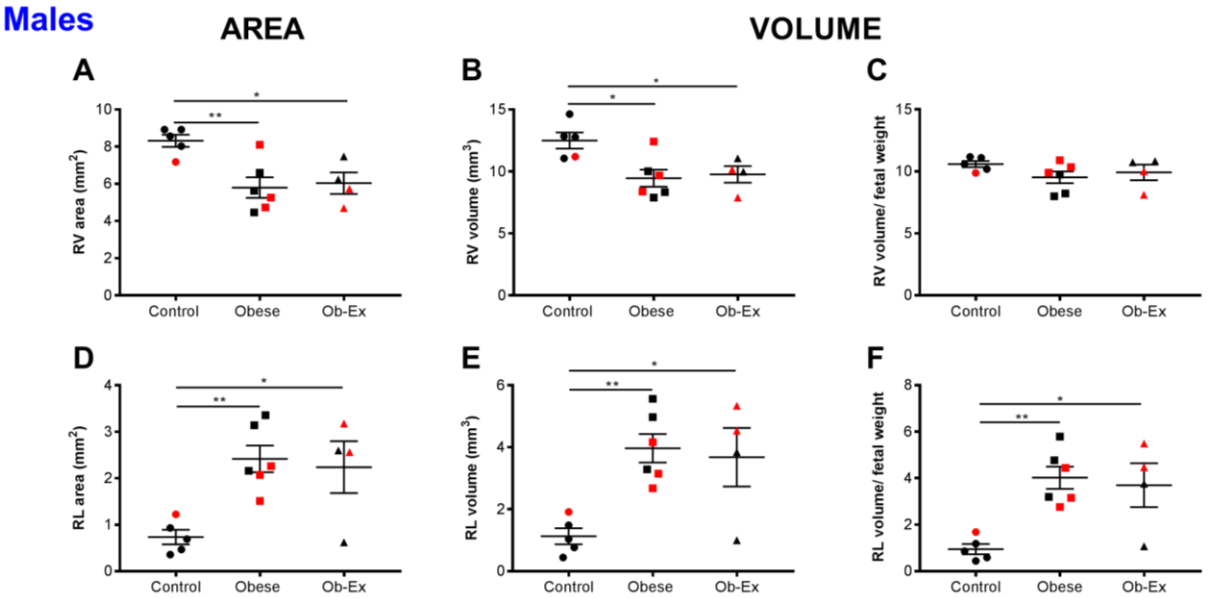


FIGURE 6.8: Area and volume of RV and RL in male E19 hearts. A) RV area B) RV volume C) RV volume relative to fetal weight D) RL area E) RL volume and F) RL volume relative to fetal weight. Red data points are those analysed hearts with 'twisted' alignment. Control *n*= 5, Obese *n*= 6 and Ob-Ex *n*= 4. One-way ANOVA with Bonferroni post-hoc test; * $p< 0.05$, ** $p< 0.01$.

Females

In the female hearts, RV area and volume were decreased in the fetuses of obese dams compared to fetuses of control dams (Figure 6.9A+B). This difference was absent when expressed relative to fetal weight (Figure 6.9C). There were no differences in RL measurements between the groups (Figure 6.9D-F). The RV: RL volume ratio was unchanged between groups (Control, 5.21 ± 1.51; Obese, 3.04 ± 0.75; Ob-Ex, 2.88 ± 0.50; $p= 0.294$).

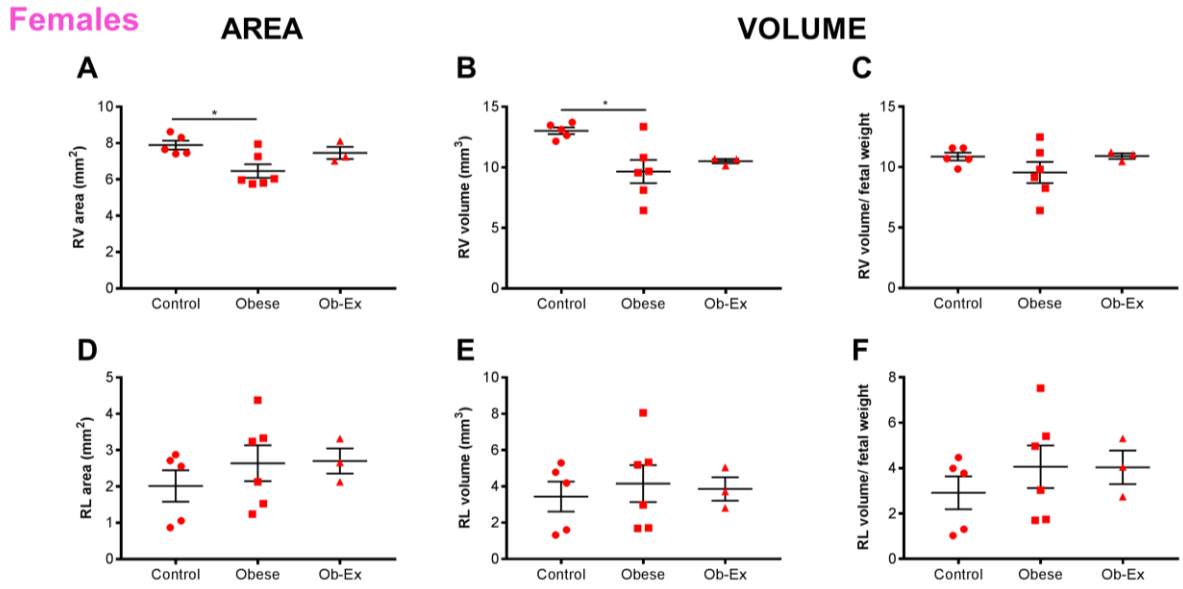


FIGURE 6.9: Area and volume of RV and RL in female E19 hearts. A) RV area B) RV volume C) RV volume relative to fetal weight D) RL area E) RL volume and F) RL volume relative to fetal weight. Red data points are those analysed hearts with 'twisted' alignment. Control *n*= 5, Obese *n*= 6 and Ob-Ex *n*= 3. One-way ANOVA with Bonferroni post-hoc test; * $p< 0.05$.

6.3.6 Lipid peroxidation-MDA assay

Whole E19 hearts were assessed for the level of lipid peroxidation (MDA). There were no measured differences in the level of MDA in the male fetal hearts (Figure 6.10A). However, no significant increase in MDA level in the female fetal hearts of obese dams compared to control, however an increase was prevented by maternal exercise (Figure 6.10B).

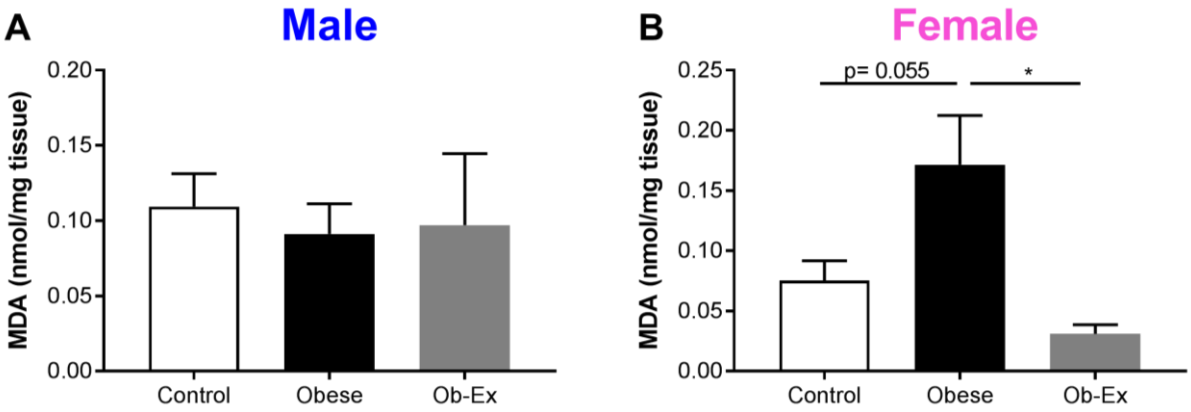


FIGURE 6.10: MDA assay in male and female E19 hearts. MDA normalised to mg of heart tissue in A) male and B) female fetal hearts. Male: Control *n*= 5, Obese *n*= 4 and Ob-Ex *n*= 4. Female: Control *n*= 5, Obese *n*= 5 and Ob-Ex *n*= 4. One-way ANOVA with Bonferroni post-hoc test; * p< 0.05.

6.4 Discussion

This chapter aimed to perform stereological analysis of the hearts from fetuses of obese dams, to assess if cardiac morphology was directly altered by an obesogenic environment during development. This was undertaken to identify possible causes for the poor cardiovascular health of offspring of obese dams in adulthood. A further aim was to establish if a maternal exercise intervention had any impact on fetal heart morphology. The findings revealed that the male fetal hearts were more affected then the female hearts. Male and female fetuses of obese dams were smaller at E19, suggesting that they were growth restricted, and this was not corrected by maternal exercise intervention. When a correction for the smaller fetal weight of the obese dams was added, it showed that the heart volume was increased in the males and this was caused by an increase in the volume of both lumens. This was not present in the female fetuses.

The second aim was to assess if oxidative stress could be a mediator for the changes in the fetal heart and to do this lipid peroxidation (MDA) was assessed in whole fetal hearts. As opposed to the cardiac stereology analysis, there was no significant increase in the amount of MDA in both the male and female fetal hearts of obese dams. The changes in cardiac structure could not be prevented by the maternal exercise intervention however the MDA level was normalised to control levels in the female fetal hearts of obese-exercised dams.

6.4.1 Altered cardiac morphology in male fetuses after exposure to maternal obesity

Despite the smaller fetal weight caused by maternal obesity, the heart area and volume were unchanged between groups. It was therefore important to correct for the difference in body size by normalising the stereological measurements by fetal weight. This adjustment revealed increased heart volume in the male fetuses of obese and obese-exercised dams. During normal fetal life, the heart should increase in size and chamber volume in proportion to body growth (Louey and Thornburg, 2005). Therefore, the heart size in the males was disproportionate to the fetal size indicating increased and possibly aberrant cardiac growth. A previous study has shown that expression of the gene *Nppa*, which is mainly associated with cardiac hypertrophy, was increased in the fetal hearts of obese dams generated in the same model (Loche 2018, personal communication). *Nppa* has been shown to suppress cardiomyocyte proliferation in the fetal sheep heart and it is hypothesised that it controls normal heart growth, in particular preventing cardiac overgrowth. Mice lacking NPRA have larger hearts at birth when compared to the wild-type controls (Knowles *et al.*, 2001). Activation of *Nppa* in the fetal

heart occurs in response to increased stretch, much like the adult heart (Jaekle *et al.*, 1995). In the fetuses of obese dams, increased expression of *Nppa* could be compensatory in an attempt to normalise cardiac growth in response to myocardial stretch.

The increase in heart volume in male offspring was driven by greater lumenal volume in both the left and right ventricles. The decreased ratios of LV: LL and RV: RL observed in the maternal obesity exposed-fetuses demonstrated an increased lumenal volume that was not proportional to ventricle wall volume. This indicated chamber dilatation without any change in ventricle wall measurements. These changes are associated with a type of cardiac hypertrophy that is known as eccentric hypertrophy (Müller and Dhalla, 2013). It has been suggested that eccentric hypertrophy can occur as a result of a reduced cardiomyocyte number, a study using forensic autopsy reports showed that hearts with eccentric hypertrophy were more likely to have fewer cardiomyocytes (Tracy, 2013). It will be important in the future to 1) assess cardiomyocyte proliferation through either Ki-67 or BrdU analysis, 2) quantify cardiomyocyte number, and 3) calculate the mononucleated/binucleated ratio. This would help define if morphological differences are due to changes in the regulation of cardiomyocyte development. There may also be rationale for analysing cardiomyocyte cell size, as both cardiomyocyte length and width are increased in eccentric hypertrophy (Heineke and Molkentin, 2006).

Volume overload is the primary cause of eccentric hypertrophy, and the molecular basis for this type of hypertrophy differs from concentric hypertrophy, which is more likely to be caused by pressure overload. Concentric hypertrophy is the enlargement and thickening of ventricular walls (Müller and Dhalla, 2013) and this more closely matches the type of hypertrophy seen in the LV from male offspring of obese dams at eight weeks of age, for which wall widths were increased (Fernandez-Twinn *et al.*, 2012). Volume overload induced-eccentric hypertrophy in the adult heart can have causes that are either physiological (e.g. pregnancy and endurance aerobic training) or pathological (e.g. chronic kidney disease, valve regurgitation and anemia) (Heineke and Molkentin, 2006; Müller and Dhalla, 2013). Further work is needed to investigate possible causes of the hypertrophy in the fetal heart. It remains to be ascertained whether the hypertrophy is pathogenic in nature, and in forthcoming studies it will be important to assess this, as pathological eccentric hypertrophy will very likely cause cardiac dysfunction (Heineke and Molkentin, 2006). It would be useful, therefore,

in future studies to perform fetal Doppler echocardiography to assess fetal cardiac function, though this may be technically challenging in the mouse.

Anemia is one mechanism for generating volume overload that can occur in the fetus. Possible causes of the anemia are red blood cell destruction as well as poor production. Maternal obesity has been shown to have a negative influence on the iron status of both the mother and the infant (Jones *et al.*, 2016). Fetal anemia results in a diminished oxygen carrying capacity to the developing tissues. The heart adapts to attempt to maintain sufficient oxygenation by increasing cardiac output through increases in venous return (preload) and ventricular filling pressures (Davey, Szwast and Rychik, 2012). There are also positive inotropic and chronotropic effects and this causes increased fetal HR. These necessary hemodynamic changes lead to enhanced myocardial stretching and cardiac enlargement/hypertrophy (Metivier *et al.*, 2000). Fetal anemia is a possible mechanism in the resulting cardiac remodelling seen in the fetuses of obese dams. Preliminary data measuring haematocrits in E19 fetal blood indicates a possible decrease in the maternal obesity exposed groups, with no protective effect of the exercise intervention (Fernandez-Twinn 2018, personal communication).

Another possible cause for fetal cardiac remodelling could lie in changes to the placental and fetal circulation. Chronic hypoxia in pregnant guinea pigs increased uterine resistance and pulsatility index, causing IUGR in the developing fetus. Hypoxia had no impact on umbilical artery Doppler indices, meaning that afterload experienced by the hearts was not altered. Diastolic filling was enhanced, indicating an increased preload mediated by increased venous return (Turan, Aberdeen and Thompson, 2017). Volume overload and increased filling pressures will lead to increased stretching in the heart, and this will trigger a hypertrophic response increasing contractile force to counter balance increased wall stretch (Mihl, Dassen and Kuipers, 2008). In the mouse model used in this thesis, placental hypoxia is thought to be present in obese dams and to be ameliorated by the exercise intervention (Fernandez-Twinn *et al.*, 2017). The cardiac morphological changes did not mirror the positive findings in the intervention group, therefore hypoxia alone may not be the driving mechanism in the observed cardiac remodelling. Hypoxia, however, is not the only cause for variations in uterine and umbilical blood flow, it can be negatively impacted by placental insufficiency (Papageorghiou and Roberts, 2005) and hypertensive disorders during pregnancy (Lee *et al.*,

2016). It would therefore be valuable to assess umbilical and uterine artery Doppler flow measurements in future studies.

6.4.2 Female fetal hearts were protected from exposure to maternal obesity

Unlike the male fetuses, all measurements of total heart area and volume were unaltered in the female fetuses of obese dams. In keeping with this, there were no changes in the ventricular or lumenal stereological measurements that were not in proportion to the smaller fetal size. Differences in the female fetuses of obese-exercised dams will be discussed in Section 6.4.3. Direct comparison between the sexes was not possible due to the different heart alignments seen between the sexes. Limitations exist within this study that might mean that true differences are being missed. The variability within the groups is greater in the female than in the male stereological data. It is, as yet, unclear why this is true. A source of the variability could be a due to the alignment of the heart, with all females appearing to have a different alignment to the males. Ideally, a more stringent criteria for an acceptable alignment for analysis should be used, however in the present study the number of available samples did not allow for this.

Chapter 5 of this thesis demonstrated that female adult offspring showed an arguably milder cardiac phenotype with a less significant degree of hypertrophy that is confined to the RV and no obvious LV systolic dysfunction. Data from this chapter, could suggest that even before birth, males are far more susceptible to the damaging effects of maternal obesity on their cardiovascular health. In Section 5.1.2, the cardioprotective properties of estrogen were discussed, and although this factor will have influence in an adult mouse, it is unlikely to be responsible for the differences seen at this fetal stage as they are not sexually mature. Therefore, in this instance it is perhaps more accurate to conclude that adaptations to adverse intrauterine exposures are different between the sexes.

6.4.3 Effect of maternal exercise intervention during an obese pregnancy on the fetal heart

The exercise intervention did not prevent the reduced fetal weight seen in maternal obesity-exposed fetuses. This was supported by neonatal offspring data (discussed in Section 3.4.2) where maternal exercise intervention did not cause any additional changes in pup weight at PND2. Stereological parameters in the male fetuses of obese-exercised dams were similar to those of the male fetuses of obese dams who were not exercised. This suggests the maternal exercise intervention could not have any impact on male fetal cardiac structure. This is

interesting as in the adult male the intervention has a striking protective effect on heart morphology and function. It is unclear how and why, despite showing similar findings in the fetal heart, the male offspring of obese dams go on to develop significant LV hypertrophy and systolic dysfunction whereas the male offspring of obese-exercised dams do not. This would be an interesting question to address in future work.

The data in the female fetus appears more puzzling; total heart area was increased in fetuses of obese-exercised dams. This was not accompanied by changes in total heart volume or even in ventricular or lumenal area measurements. The lack of significant differences in these parameters may be due to a lack of power in that group, as unfortunately only a sample size of three was possible. It is, therefore, an unclear finding that would need further investigation.

6.4.4 Oxidative stress as a mediator for poor cardiovascular health

Placental hypoxia is present in the obese dams during pregnancy (Fernandez-Twinn *et al.*, 2017) but it has not yet been determined if this insult will be transferred to the fetus. Previous work has assessed the level of expression of antioxidant defence genes in the fetal heart; data is shown in Appendix AP3. Catalase and superoxide dismutase 2 are two of the critical enzymes in the antioxidant defence network against oxidative stress damage mediated by reactive oxygen species (ROS) (Birben *et al.*, 2012). Both were increased in male and female fetal hearts of obese and obese-exercised dams. Nuclear factor erythroid 2–related factor 2 is a transcription factor that is a major regulator of antioxidant defence (Ma, 2013), and was similarly increased in hearts of male and female fetuses of obesogenic diet-fed dams. Glutathione peroxidase 3, like catalase, acts in the defence against ROS by eliminating hydrogen peroxide (Birben *et al.*, 2012), and this was elevated with maternal obesity in a sex-dependent manner, where just the males showed an elevated expression. Previous studies have also shown a lower expression of glutathione peroxidase in female animals (Kander, Cui and Liu, 2017).

Despite the elevated expression of antioxidant defence genes in male and female fetal hearts of obese dams (Appendix AP3), lipid peroxidation was not significantly elevated in the fetal hearts of obese dams. Secondly, in the female fetuses, maternal exercise appears to be protective against lipid peroxidation in these hearts. This suggests that although antioxidant defence has been triggered, these defensive systems have not been overcome, therefore oxidative stress was not yet present if the obese dam was exercised during pregnancy or if the fetus was male. However, there are other consequences of oxidative stress (e.g. DNA

damage) and these need to be assessed to determine if male fetuses and fetuses of obese-exercised dams are fully protected against oxidative stress.

The lipid peroxidation findings do not mirror the changes in the fetal heart structure. Despite significant cardiac morphological changes in the male fetuses of obesogenic diet-fed dams, no changes in lipid peroxidation between male groups were seen. On the other hand, the expression profile of antioxidant defence genes was in agreement with cardiac changes in the male fetus indicating it was a potential mediator. The female fetal hearts of obese dams had no significant increase in lipid peroxidation (MDA) level, and in contrast to the males this was accompanied with no cardiac morphological changes. All measured antioxidant defence genes, except glutathione peroxidase 3, were also upregulated in the female fetal hearts despite no cardiac changes. Many studies have shown that females have an apparent lower susceptibility to oxidative stress damage (Marotti *et al.*, 2010; Kander, Cui and Liu, 2017), and although the MDA data did not fully show this, it still may explain the prevention of the cardiac remodelling.

6.4.5 Conclusions

Maternal obesity caused cardiac remodelling in the E19 heart in a sex-dependent manner. The sex of the fetus determined how the fetus adapted to the developmental stressor of an obesogenic environment. These differences could explain why the cardiovascular phenotype in the adult offspring is so different between the sexes. The maternal exercise intervention was not successful at preventing the cardiac structural changes, therefore the mechanism by which the maternal exercise intervention prevents cardiac dysfunction in the male adult offspring is unknown.

6.4.6 Summary of key findings

Results from this chapter has shown that:

- Fetuses of obesogenic diet-fed dams were smaller at E19 and may be growth restricted.
- Hearts from male fetuses of obesogenic diet-fed dams displayed signs of chamber dilatation and possible eccentric hypertrophy.
- Female fetal heart morphology appeared to be protected from the adverse exposure of maternal obesity.

- Maternal exercise did not appear to protect the male fetal hearts from altered stereological measurements and further work is needed to determine the full effect on female fetal hearts.

- Lipid peroxidation was not significantly increased in the fetal hearts of obese dams despite elevated oxidative stress markers.

- The maternal exercise intervention reduced lipid peroxidation in female fetal hearts when compared to the female fetuses of non-exercised obese dams, even though the intervention did not alter oxidative stress markers.

7. General Discussion

This growing field of DOHaD research has transformed our understanding of health and disease by demonstrating the power of nutrition in influencing health across generations. The health legacy of an individual can be pre-determined before birth and in the earliest days of life by the intrauterine and neonatal environment. The growing prevalence of maternal obesity in today's world is of huge concern, in particular with respect to the impact of the so-called 'programmed' disease on the next generation. Strategies aiming to break the cycle and abate the burden of cardiometabolic disease need to be developed. In-depth studies need to be carried out to identify translatable interventions, in order to incorporate evidence-based interventions into clinical practise.

This thesis sought to test the effectiveness of a maternal exercise intervention in preventing the unwanted cardiovascular changes that have previously been shown to arise in the male offspring born to obese dams. In undertaking this study it was important to define the *in utero* environment in the obese dams, and how this was altered by the intervention, in order to be able to identify drivers of programming mechanisms. There is a lack of studies that investigate cardiovascular outcomes in the female offspring of obese dams. Therefore, this thesis also aimed to fill the current knowledge gap about this often under-represented sex in programming studies. The final aim of this thesis was to identify the immediate consequences of maternal obesity on the fetal heart, and also determine the influence of the maternal exercise intervention. Defining these changes would improve understanding of how and why the poor cardiovascular health in the offspring occurs, as well as trying to address the sex differences in these cardiovascular phenotypes. The findings in the offspring are summarised in Figure 7.1.

7.1 Sex differences in the response to exposure to maternal obesity

When this thesis investigated the cardiovascular health of both male and female offspring, it became clear that significant sex differences existed in the programming by maternal obesity. The differences it highlighted will be explored in the following section. Male fetuses of obese dams had enlarged heart to body ratios, which was caused by increases in both lumenal volumes after adjustment for the smaller bodyweights of these growth-restricted fetuses. Despite the female fetuses of an obese pregnancy also being growth restricted, they were protected against cardiac remodelling during heart development. This highlights a difference

between the sexes in their response and ability to adapt to an adverse intrauterine obesogenic environment.

Cardiac hypertrophy in the offspring of obese dams appears to affect different ventricles depending on sex; the LV hypertrophy was present in the male offspring while only the RV was impacted in the female offspring. The cause of hypertrophy in each ventricle may be distinct and the mechanisms should be explored in further work. Nevertheless, it has not yet been determined if the male offspring also had RV hypertrophy and this remains an important question to address. Data from this thesis indicates that RV hypertrophy is expected to be present in the males as the fetal heart analysis showed that the right side of the heart was equally as affected as the left side. Secondly, the offspring were hypertensive and this could be further complicated by pulmonary hypertension (as discussed in Section 5.4.3), which will inevitably impact the RV. Using the automated cell area method developed during this PhD, this analysis can now be carried out with relative ease.

LV hypertrophy appeared to be associated with LV systolic dysfunction and fibrosis since these outcomes always occurred together. Unlike the male offspring, the female offspring appear protected from LV dysfunction, despite haemodynamic stress acting on their hearts caused by hypertension, and indications of an adverse expression profile of myofilament contractile proteins. Nonetheless, cardiac function does need to be assessed in the RV of the female hearts because it is this side of the heart that has been affected by the hypertrophy. The RV remodelling in the female hearts did not cause cardiac fibrosis unlike the cardiac remodelling seen in the male offspring, possibly indicating it is not as severe. The female offspring appear protected from the most adverse cardiovascular outcomes programmed by maternal obesity. This divergence in outcomes between male and female offspring is observed in young adult life. A suboptimal early-life environment increases propensity for age-associated disease (Tarry-Adkins and Ozanne, 2017), and programmed phenotypes typically worsen or become more pronounced with age, consequently it will be important to establish if females remain protected in later life.

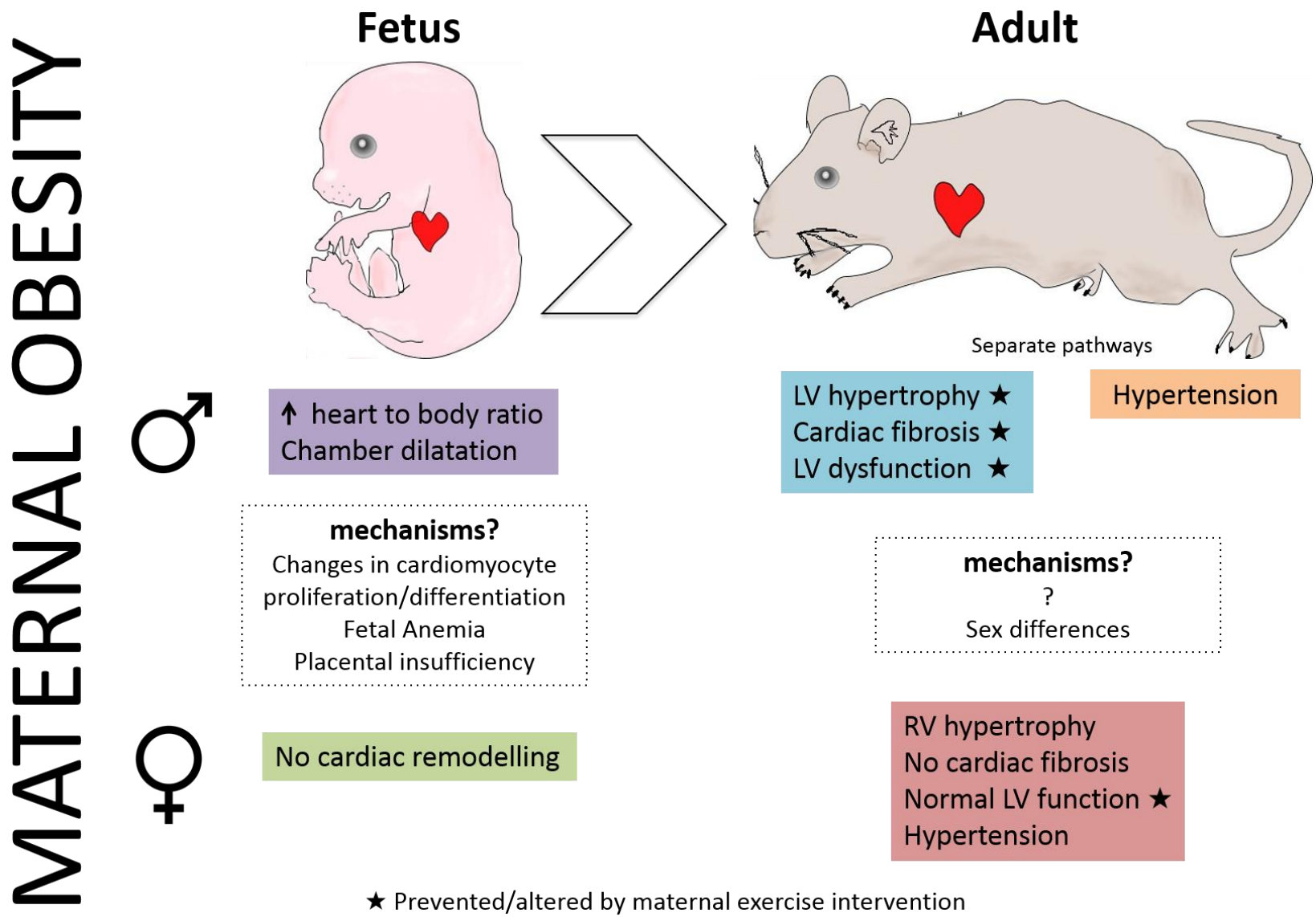


FIGURE 7.1: Summary of the cardiovascular findings in the offspring of obese dams.

7.2 Similar effects of exercise intervention on the offspring hearts

Despite different cardiovascular phenotypes that occurred between sexes in response to maternal obesity, the maternal exercise intervention altered cardiac function in both sexes. The effects of the exercise intervention are visualised in Figure 7.1 through the use of a star symbol. The exercise intervention in the obese dams increased offspring cardiac contractility, which in the males was possibly mediated by sympathetic activated inotropy. Previous studies using Langendorff data has shown that male offspring of non-exercised obese dams had sympathetic dominance accompanying *ex vivo* cardiac dysfunction. To explore the hypothesis of sympathetic activated increases in inotropy, future work could use a Langendorff isolated heart perfusion set up to assess the responsiveness of the offspring hearts to sympathetic (isoprenaline) and parasympathetic (carbachol) agonists. In the males, this increased contractility only acted to prevent the maternal obesity-induced cardiac dysfunction, as they had to overcome a deficit in cardiac function that was present in the offspring of non-exercised obese dams. This deficit in cardiac function shown in the male offspring of obese dams was likely a result of the altered hemodynamic load caused by increased SBP. In the females, this increased contractility resulted in systolic function parameters that were increased even when compared to the offspring of control dams.

A short exercise intervention undertaken during an obese pregnancy in males was able to impact the cardiac health of the offspring in early adulthood, despite there being no protective effect seen in fetal life. This presents strong evidence for the importance of intervention studies undertaking long-term follow up in the offspring, in order to be able to fully assess the potential value of the intervention. It could suggest that even the most disappointing study, showing no positive outcomes of the intervention on the fetus or neonate, could still be advantageous to those offspring in adulthood. This finding is particularly relevant to human studies where primary fetal outcomes are often not met.

7.3 Unanswered questions and future directions

7.3.1 What causes the cardiac remodelling in the fetal heart?

An eccentric hypertrophy-like phenotype was seen in the male fetal hearts of obese dams, with chamber dilatation in both ventricles. The mechanism underlying the fetal cardiac changes cannot involve factors that were corrected by the maternal exercise intervention.

Modifications in cardiomyocyte proliferation or differentiation, that will respectively alter the number and maturation of the cardiomyocytes, could be a possible cause. Further histological and immunohistochemical analyses should be carried out in the fetal heart to answer this question. Another mechanism to investigate is fetal anemia, a cause of fetal heart failure that results in adaptive cardiac remodelling, leading to the abnormal enlargement of the heart (Davey, Szwast and Rychik, 2012). In order to assess fetal anemia, fetal blood haematocrits should be measured at the point of *post mortem*. It is also not yet clear how the female fetal hearts can be protected from the cardiac remodelling caused by these potential mechanisms. It is also important to establish if this cardiac remodelling is accompanied by cardiac dysfunction, and this can be assessed by fetal Doppler echocardiography. The reliance of the fetal heart on the maternal circulation means that changes in umbilical and uterine pulsatility indices, often caused by placental insufficiency, are further possible causes of fetal cardiac remodelling.

7.3.2 Why do the cardiac changes that occur in fetal life develop into the different cardiovascular phenotypes in the adult?

Male fetuses of obese dams had fetal cardiac remodelling, which the maternal exercise intervention did not appear to prevent, and yet these fetuses will grow up and have very different cardiovascular phenotypes in early adulthood. Male fetuses of obese dams become adults who have LV hypertrophy, cardiac fibrosis, and systolic dysfunction. On the other hand, the offspring from the intervention group were protected against these cardiac changes, despite sharing the same fetal heart changes. The maternal exercise intervention ends on E17 of pregnancy, which is before the fetal heart analysis was undertaken, so the intervention period is short and yet it appears to still be able to influence the offspring many weeks later. Epigenetic mechanisms that allow the intervention offspring to avoid these unfavourable changes should be explored.

The neonatal period is an important developmental window in the mouse and therefore programming of adverse offspring outcomes can still occur during this time. The intervention did not continue after birth and was solely carried out during pregnancy. The positive effects of the exercise intervention in the dam is likely to be transient, it is currently unknown if the exercise intervention during pregnancy can still benefit the offspring during this period. This remains an important question to answer.

7.3.3 What mechanisms underlie the programmed sex differences in the offspring?

In both fetal and young adult life, the female offspring of obese dams appear to be protected from the worst of the adverse programming effects. They did not have fetal cardiac remodelling and the cardiovascular phenotype at eight weeks of age was also, arguably, milder. The females had RV, and not LV, hypertrophy that was not complicated by fibrosis and there was no LV systolic dysfunction. A limitation of this thesis was that the experiments in the male and female offspring were undertaken at different stages of the PhD, and therefore direct comparisons were not fully possible. In future studies, male and female littermates should be compared and this will allow statistics to analyse for true sex differences, while controlling for other confounding factors. Possible mechanisms that can mediate these sex differences should be explored; including epigenetics, difference in circulating levels and sensitivity to metabolic hormones, and finally the cardioprotective effects of estrogen (Dearden, Bouret and Ozanne, 2018). This is a challenging question to fully answer. To assess the role of estrogen on the cardiovascular outcomes in the adult mouse, it would be possible to perform an ovariectomy on the female offspring to remove the influence of estrogen.

7.3.4 What programming mechanisms are involved in the development of offspring hypertension?

Maternal obesity, in our model, programmed hypertension in the offspring. Due to significant sex differences, this cardiovascular outcome was the only measure that was present in both sexes. The intervention undertaken in this PhD did not prevent offspring hypertension, therefore the mechanism involved could not have been corrected by the exercise intervention. Furthermore, in the males, the programming pathway that mediates offspring hypertension must be divergent to the pathway controlling cardiac hypertrophy and dysfunction. Exercise did not lower body fat mass in the dam and, in keeping with this, they remained hyperleptinemic. Leptin has been shown to be involved in the programming of hypertension in the offspring (Samuelsson *et al.*, 2010, 2016). The postnatal leptin surge should be tracked in the neonates, to establish if the exaggerated surge that is seen with maternal obesity continues to be exaggerated after the maternal exercise intervention. It is important to identify the mechanism involved in order to be able to develop a new intervention that can target this particular programmed effect.

To be able to fully target the cardiovascular health of the offspring exposed to maternal obesity, another intervention needs to be combined with the exercise intervention.

Behavioural interventions, used in the UPBEAT study, combine exercise with a dietary intervention that recommended a low glycemic index (GI) diet (Poston *et al.*, 2015). Another potential intervention is metformin administration during pregnancy; a previous study suggests it could be a good candidate since prenatal metformin in high fructose diet-fed dams prevented hypertension in HFD diet-fed offspring (Tain *et al.*, 2018). Finally, antioxidants could be employed alongside exercise to address the oxidative stress that may be a potential programming factor in the dam.

7.4 Concluding remarks

The deleterious influence of an obesogenic environment on the offspring's cardiovascular health is evident before birth. Maternal obesity disrupted heart development of male, but not female, fetuses. A maternal exercise intervention during pregnancy could not prevent this disadvantageous change. In young adulthood, male offspring developed more significant deficits in their cardiovascular health. Female offspring had more favourable maternal obesity-programmed cardiovascular outcomes, as they demonstrated a greater ability to adapt to a harmful *in utero* environment. The exercise intervention during pregnancy in the obese dams did prevent, to a certain degree, this detrimental progression to poor cardiovascular health. There is a need for combination interventions to fully target the offspring's poor cardiovascular health. In developmental programming, it is no longer appropriate to base the success of an intervention study on short-term outcomes. This thesis provides evidence for an intervention that can have long-term beneficial effects despite initially showing some shortfall in preventing disadvantageous short-term outcomes.

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Appendix

AP1 Cardiac calculations used in Vevo770 software

$$ESV\ (\mu l) = \left(\frac{7}{(2.4 + LVID;diastole)}\right) \times LVID;diastole^3$$

$$EDV\ (\mu l) = \left(\frac{7}{(2.4 + LVID;systole)}\right) \times LVID;systole^3$$

$$stroke\ volume\ (SV)(\mu l) = End\ diastolic\ volume\ (EDV) - End\ systolic\ volume\ (\mathrm{ESV})$$

$$Ejection\ fraction\ (\%) = \frac{SV}{EDV} \times 100$$

$$Fractional\ shortening\ (\%) = \frac{end\ diastolic\ diameter - end\ systolic\ diameter}{end\ diastolic\ diameter}$$

$$Cardiac\ output\ (\mu l/min) = SV\ \times\ Heart\ rate$$

$$LV\ mass\ (mg) = 1.053 \times ((LVID;diastole + LVPW;diastole + IVS;diastole)^3 - LVID;diastole^3)$$

AP2 Post weaning dam GTT with three groups (Control, Obese and Ob-Ex)

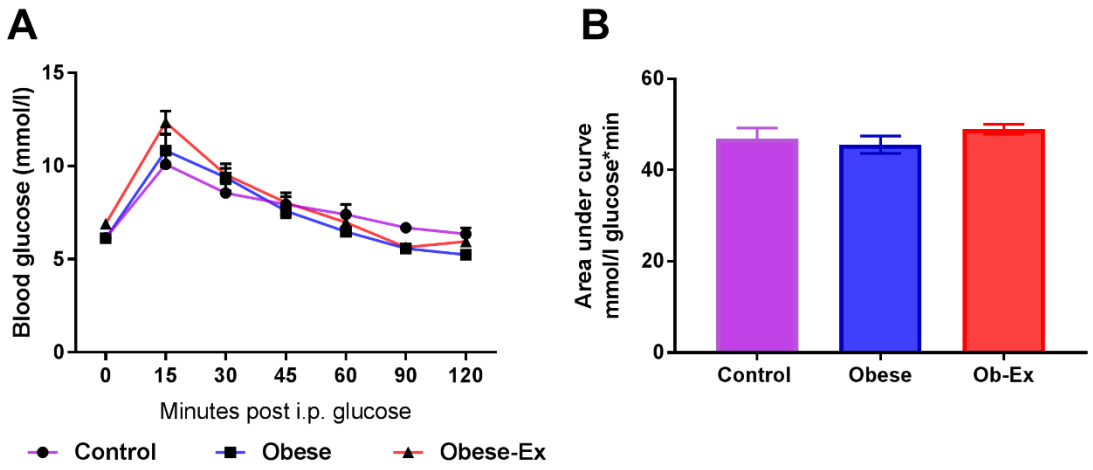

FIGURE 1: Maternal GTT at weaning (Control, Obese and Ob-Ex). Data kindly provided by Dr Laura Kusinski and Dr Lisa Nicholas. Control *n*= 15, Obese *n*= 10 and Ob-Ex *n*= 5.

AP3 E19 oxidative stress genes in E19 heart

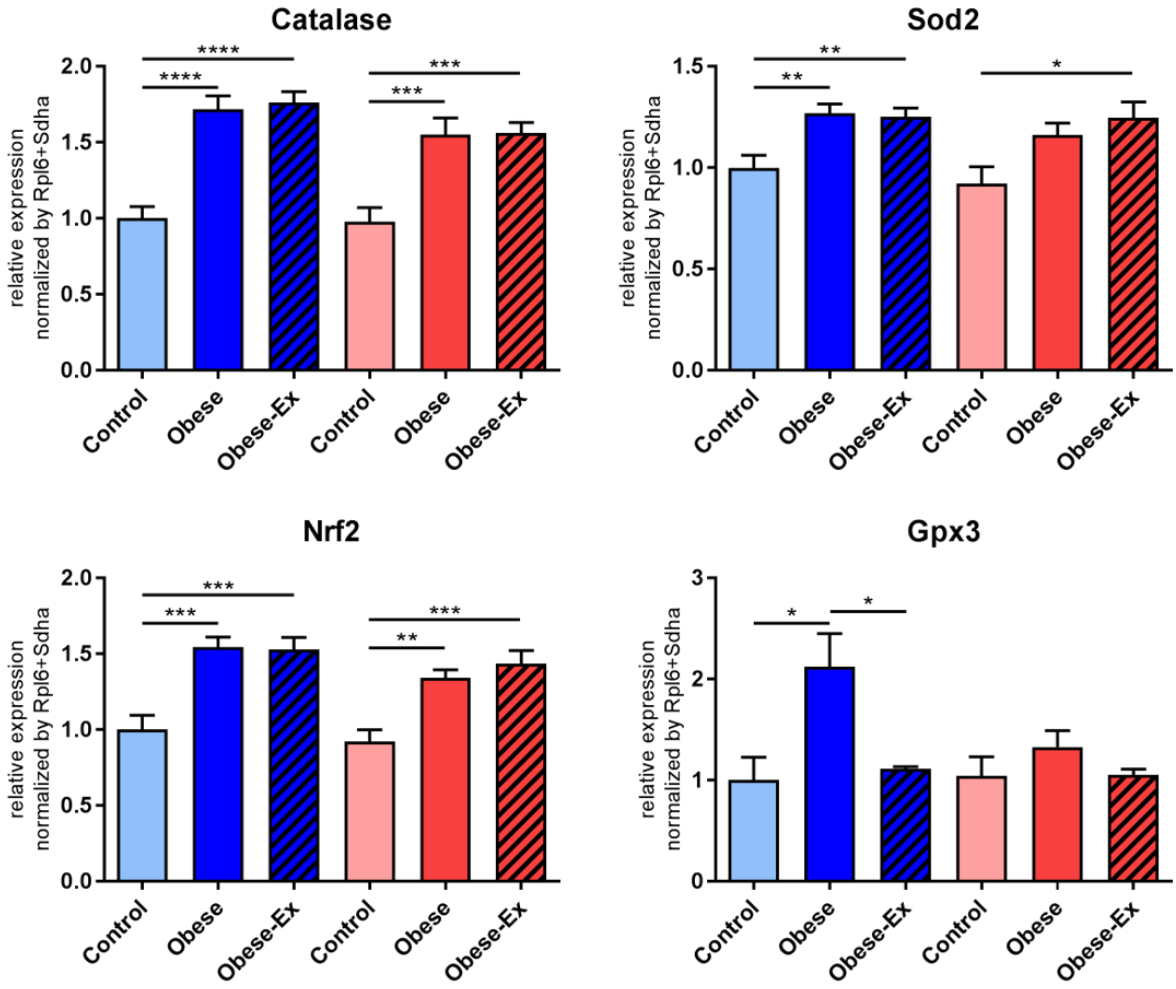

FIGURE 2: Expression of antioxidant defence genes in E19 hearts. mRNA gene expression was assessed in the heart tissue of male and female foetuses of control, obese and obese-exercised dams. Data kindly provided by Dr Daniela de Barros Mucci. *Cat*- Catalase, *Sod2*- Superoxide dismutase 2, *Nrf2*- Nuclear factor erythroid 2–related factor 2 and *Gpx3*- Glutathione peroxidase. Expression normalised to *Rpl6*- Ribosomal protein L6 and *Sdha*- Succinate dehydrogenase complex, subunit A. Male E19: Control *n*= 8, Obese *n*= 8 and Ob-Ex *n*= 7. Female E19: Control *n*= 7, Obese *n*= 7 and Ob-Ex *n*= 7. One-way ANOVA with Tukey post-hoc test, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ and **** $p< 0.0001$.